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In vitro evaluation, biodistribution and scintigraphic imaging in mice of radiolabeled anthrax toxins

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Abstract

Introduction—There is a lot of interest towards creating therapies and vaccines for *Bacillus anthracis*, a bacterium which causes anthrax in humans and which spores can be made into potent biological weapons. Systemic injection of lethal factor (LF), edema factor (EF), and protective antigen (PA) in mice produces toxicity and this protocol is commonly used to investigate the efficacy of specific antibodies in passive protection and vaccine studies. Availability of toxins labeled with imaggable radioisotopes would allow to demonstrate their tissue distribution after intravenous injection at toxin concentration that are below pharmacologically significant to avoid masking by toxic effects.

Methods—LF, EF and PA were radiolabeled with ¹⁸⁸Re and ^{99m}Tc and their performance in vitro was evaluated by macrophages and CHO cells toxicity assays and by binding to macrophages. Scintigraphic imaging and biodistribution of IV injected ^{99m}Tc- and ¹²³I-labeled toxins was performed in BALB/c mice.

Results—Radiolabeled toxins preserved their biological activity. Scatchard-type analysis of the binding of radiolabeled PA to the J774.16 macrophage-like cells revealed 6.6×10^4 binding sites per cell with a dissociation constant of 6.7 nM. Comparative scintigraphic imaging of mice injected intravenously with either ^{99m}Tc- or ¹²³I-labeled PA, EF, and LF toxins demonstrated similar biodistribution patterns with early localization of radioactivity in the liver, spleen, intestines and excretion through kidneys. The finding of renal excretion shortly after IV injection strongly suggests that toxins are rapidly degraded which could contribute to the variability of mouse toxicogenic assays.

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Biodistribution studies confirmed that all three toxins concentrated in the liver and the presence of high levels of radioactivity again implied rapid degradation in vivo.

Conclusions—The availability of ^{188}Re and $^{99\text{m}}\text{Tc}$ -labeled PA, LF and EF toxins allowed us to confirm the number of PA binding sites per cell, to provide an estimate of the dissociation constant of PA for its receptor, and to demonstrate tissue distribution of toxins in mice after intravenous injection.

Keywords

anthrax; protective antigen; lethal factor; edema factor; 188-Rhenium; scintigraphic imaging

INTRODUCTION

There is a lot of interest towards creating therapies and vaccines for *Bacillus anthracis*, a bacterium which causes anthrax in humans and which spores can be made into potent biological weapons. We are interested in radiolabeling the components of the tripartite toxin secreted by *B. anthracis*, namely: protective antigen (PA), named for its ability to elicit protective immunity, lethal factor (LF) which is a zinc-dependent protease that cleaves mitogen-activated protein kinase kinase, and edema factor (EF) - an adenylate cyclase capable of impairing host defenses [1]. Systemic injection of LF, EF, and PA in mice produces toxicity and this protocol is commonly used to investigate the efficacy of specific antibodies in passive protection and vaccine studies [2–5]. Although this assay is not physiological considering the pathogenesis of anthrax, it provides a means to test some aspects of toxin function. Availability of toxins labeled with imaggable radioisotopes would allow to demonstrate their tissue distribution after intravenous injection. The important advantage of using radiolabeled toxins would also be the fact that since imaging is performed at toxin concentration that are below pharmacologically significant, these observations would show metabolism patterns not masked by toxic effects. We have recently radiolabeled PA and LF with ^{188}Re in order to study the binding of these toxins to macrophages in vitro in the presence of PA-specific monoclonal antibody [6]. Here we investigate in vitro and vivo behavior of ^{188}Re -or $^{99\text{m}}\text{Tc}$ -labeled LF, EF, and PA which provide new insights into the biological properties of these toxins.

MATERIALS AND METHODS

Toxins and cells

Recombinant protective PA, LF and EF expressed in *E. coli* as described [7] were obtained from Wadsworth Laboratories, NYS Department of Health (Albany, NY). Murine J774.16 macrophage-like cells were grown in DMEM supplemented with 10% FCS, 10% NCTC-109 and 1% nonessential amino acids. The J774.16 cell line was originally obtained from Dr. Barry Bloom (Boston, MA) and has been extensively used in our laboratories in phagocytosis assays [8].

Radioisotopes

$^{99\text{m}}\text{Tc}$ in form of sodium pertechnetate $\text{Na}^{99\text{m}}\text{TcO}_4$ was procured from GE Healthcare; ^{123}I in form of sodium ^{123}I -iodide - from MDS Nordion (Canada). ^{188}Re in form of sodium perrhenate $\text{Na}^{188}\text{ReO}_4$ was eluted from $^{188}\text{W}/^{188}\text{Re}$ generator (Oak Ridge National Laboratory, Oak Ridge, TN). Radiolabeling of LF, EF, and PA was performed as described in [6].

Macrophage toxicity assay

To investigate whether labeling with ^{188}Re affected the biological properties of the various toxins, the toxicity of a ^{188}Re -labeled lethal toxin towards macrophages was assessed. Lethal

toxin (LeTx) is a mixture of PA and LF. ^{188}Re -labeled lethal (^{188}Re -LeTx) is a mixture of ^{188}Re -PA and LF. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, St. Louis, MO) was dissolved at 5 mg/mL in sterile PBS at room temperature. The solution was sterilized by passage through a 0.22 μm filter and stored in a dark bottle at 4°C. The MTT assay involves the oxidation of MTT to an insoluble pigment by live cells. J774.16 macrophage-like cells (6×10^4) were incubated in a 96-well plate with 100 ng of ^{188}Re -LeTx for 4 hours at 37°C. Unlabeled LeTx or only ^{188}Re -PA were used as controls. In a dose response experiment 12.5 – 400 ng of ^{188}Re -LeTx were used. A 25 μL volume of 5 mg/mL stock-solution of MTT was added to each well and after 2 h incubation at 37°C, 100 μL of the extraction buffer (12.5% SDS, 45% DMF) was added and cells were incubated overnight at 37°C. Absorbance was measured at 570 nm (Labsystem Multiskan, Franklin, MA).

CHO cells toxicity assay

In combination with PA, EF (an adenylate cyclase) is activated by calmodulin to catalyze the synthesis of cAMP in host cells [9]. Edema toxin (PA + EF, EdTx) induces an increase in the intracellular cAMP levels in sensitive cells. Chinese hamster ovary (CHO) cells were maintained in DMEM supplemented with 10% heat inactivated fetal bovine serum, 1% nonessential amino acids and 10% NTCC. CHO cells (10^5) were incubated with various dilutions of ^{188}Re -EdTx (PA and ^{188}Re -EF mixture) for 2 h at 37°C. Total cAMP levels were measured using the cAMP Biotrak enzyme immunoassay (EIA) system as described by the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ).

Binding of labeled toxins to macrophages

J774.16 cells were washed twice and re-suspended in 1 mL PBS at a density of 2.8×10^6 cells/mL. The binding studies were done in low retention siliconized microcentrifuge tubes (Fischer Scientific, Pittsburgh, PA). The microcentrifuge tubes were blocked overnight with 1% BSA to prevent non-specific binding of protein to the plastic. To determine the PA receptor density in J774.16 cells, ^{188}Re -PA was added to the tubes in the increasing amounts (0.28 – 1.92 nM). The cells were incubated for 1 hr at 4°C and the radioactivity measured using a gamma counter. Macrophages were collected by centrifugation at 1,200 rpm at 4°C for 6 min and radioactivity counts were measured. From the total and bound radioactivity we inferred the amount of toxin binding and used Scatchard-type analysis [10] to calculate the toxin dissociation constant K_D and the number of PA-binding receptors per cell.

In another series of experiments, we measured the binding of LF toxin to macrophages incubated with and without PA. The cells were incubated at 4°C for 1 hr with ^{188}Re -LF (0.28 – 1.92 nM), and in some experiments, a large molar excess (20 nM) of unmodified or DTT-treated LF was also added to the cells. The binding of ^{188}Re -LF to normal macrophages was calculated as the ratio of activity in the pellet versus activity in the tube before collecting the cells and was expressed as a percentage. For assessing the binding of LF to macrophage-bound PA, cells were first incubated with increasing amounts (0.28 – 1.92 nM) of "cold" PA for 1 hr at 4°C, followed by addition of equimolar amounts of radiolabeled LF (1:1), with an additional incubation for 1 h at 4°C. Radioactivity was measured and percent binding was calculated as described above.

Scintigraphic imaging and biodistribution of $^{99\text{m}}\text{Tc}$ - and ^{123}I -labeled toxins

Initially we investigated the in vivo localization of $^{99\text{m}}\text{Tc}$ -labeled toxins. For these studies, $^{99\text{m}}\text{Tc}$ was utilized instead of ^{188}Re because it has superior imaging characteristics - the abundance of its 140 keV photons is 89% versus 15% for 155 keV photons emitted by ^{188}Re . Tc and Re metals are chemical analogues which react with proteins in a similar way. Toxins were radiolabeled with $^{99\text{m}}\text{Tc}$ as described above. All animal studies were carried out in accordance with the guidelines of the Albert Einstein College of Medicine Institute for

Animal Studies. Five groups of 3 female BALB/c mice (NCI) were injected IV with: 200 μCi (4.5 μg) $^{99\text{m}}\text{Tc}$ -PA; 200 μCi (4.5 μg) $^{99\text{m}}\text{Tc}$ -LF; 200 μCi (4.5 μg) $^{99\text{m}}\text{Tc}$ -EF; 40 μCi (1.5 μg) $^{99\text{m}}\text{Tc}$ -LF co-injected with 4.5 μg "cold" PA; or 40 μCi (1.5 μg) $^{99\text{m}}\text{Tc}$ -EF co-injected with 4.5 μg "cold" PA. At 1 and 3 hr post-injection the animals were anesthetized with Isoflurane and imaged for 1 or 2 min on a gamma-camera (Siemens) equipped with ICON image-processing software. At 24 h post-injection the animals were sacrificed, their organs removed, blotted to remove excess blood, weighed and counted in a gamma counter. The percentage of injected dose of toxin per gram of organ tissue (ID/g, %) was calculated.

To confirm the biodistribution results, the experiment was repeated with ^{123}I -labeled toxins. PA, LF and EF were labeled with ^{123}I using Iodobeads iodination reagent (Pierce Biotechnology, Rockford, IL) by incubating 100 μg of each toxin with two Iodobeads and 50 μL (2 mCi) of sodium ^{123}I -iodide in PBS for 15 min at room temperature. Iodinated toxins were purified from unreacted ^{123}I -iodide on Centricon-30 microconcentrators, diluted with PBS and imaging and biodistribution study was performed as above.

Statistical analysis

Student's t-test for unpaired data was employed to analyze differences in the percent survival of macrophages or CHO cells treated with radiolabeled LeTx or EdTx versus unlabeled ones and in the organ uptake in the biodistribution studies. Differences were considered statistically significant when P values were < 0.05 . The binding data for the Scatchard plot were analyzed by linear regression (Prism software, GraphPad, San Diego, CA).

RESULTS AND DISCUSSION

The specific activity of labeled toxins was approximately 10 mCi/mg, which was sufficiently high for utilization in the variety of biological experiments. To assess if labeling with ^{188}Re affected the biological properties of toxins, we assayed the toxicity of radiolabeled PA followed by addition of unmodified LF for murine macrophages using the MTT assay. Addition of ^{188}Re -labeled lethal toxin (^{188}Re -LeTx) in the form of ^{188}Re -PA and LF mixture to cell monolayers in increasing doses resulted in a significant reduction in cell viability. The effect of ^{188}Re -LeTx on the cells was dose dependent in the same way as for unlabeled LeTx (Fig. 1a). We considered the possibility that the ^{188}Re emissions would be contributing to cellular death. Hence, we repeated the cellular assay with ^{188}Re -PA alone and no effects on cells were noted (Fig. 1b).

The preservation of biological activity for EF after labeling with ^{188}Re was assessed by treating CHO cells with ^{188}Re -labeled edema toxin (^{188}Re -EdTx, PA plus ^{188}Re -EF) and measuring total cAMP levels in treated cells. No difference was noted in total cAMP levels for CHO cells treated with ^{188}Re -EdTx and unlabeled EdTx (Fig. 1c). Thus, radiolabeling PA and EF with ^{188}Re did not have an effect on the biological activity of LeTx and EdTx, respectively.

As radiolabeling allows to trace the proteins in vitro and in vivo, we used the labeled toxins in several experiments. First, we measured the binding of ^{188}Re -PA to the J774.16 macrophages. For binding studies and calculation of the number of binding sites we used the technique described in the classical paper by Lindmo et. al. [10]. Previously we used this technique for the calculation of the number of binding sites on cryptococcal cells [11] and on melanin in the melanoma cells [12]. The specific activity of 10 mCi/mg allowed us to use picomolar concentrations of labeled toxins while the counts per sample were at least 10,000 cpm due to the contribution of high energy beta-particles emitted by ^{188}Re to the counting rate. The concentrations of labeled toxins used in the binding assays were also below toxic levels as per macrophage and CHO cells toxicity assays. The binding data are presented as Scatchard plot (Fig. 2a). From these data, we calculated the number of the PA-binding receptors as $6.6 \times$

10^4 per macrophage cell, taking into consideration the fact that PA forms heptamers on the surface of the cells, and a dissociation constant of 6.7 nM. Prior studies of macrophage cell lines using different techniques have measured 3×10^4 receptors per cell [2]. Our K_D value in low nanomolar range is in good agreement with the 0.9 nM value measured by Escuyer and Collier for the binding of radioiodinated PA to CHO-K1 cells [13]. The difference between the measured K_D 's may reflect subtle differences in expressed PA and/or species differences in receptor structure. Nevertheless, our measured number confirms the literature data by independent technique and is consistent with the notion that ^{188}Re -PA behaved similarly to unmodified PA molecule.

In another series of experiments, we assessed the binding of ^{188}Re -LF to normal macrophage cells and to macrophage-bound PA. The binding of ^{188}Re -LF to macrophage-bound PA was more than two fold higher than that to the non-PA-treated cells (Fig. 2b), in agreement with the data on LF and EF binding to PA heptamer on the cell surface, which then carries them into the cell. However, our data also shows significant binding of labeled LF to the cells in the absence of PA suggesting the existence of other mechanisms for LF binding to these cells (Fig. 2b).

Systemic administration of LF, EF, and PA toxins in mice produces toxicity and this protocol is commonly used to investigate the efficacy of specific antibodies in passive protection and vaccine studies [2–5]. Although this assay is not physiological considering the pathogenesis of anthrax, it provides a means to test some aspects of toxin function. The availability of labeled toxins allowed us to scintigraphically evaluate the preferential localization of toxin proteins injected into mice. Toxins were labeled with $^{99\text{m}}\text{Tc}$ using the same protocol as for labeling with ^{188}Re . Though ^{188}Re can be successfully imaged with a gamma camera from the emission of 155 keV photons with 15% abundance, $^{99\text{m}}\text{Tc}$ is a more suitable radionuclide for imaging applications because it has a much higher photon flux. The biodistribution studies were done with trace amounts of labeled compounds, well below lethal concentrations: a small dose (4.5 μg of PA and 1.5 μg of either $^{99\text{m}}\text{Tc}$ -LF or $^{99\text{m}}\text{Tc}$ -EF) was chosen to monitor the distribution of a compound in the tissues while avoiding any obvious toxic effects in mice. According to the detailed study by Moayeri et. al. [3] of anthrax toxins effects on inbred mice, 10 μg of toxins did not have any effect on sensitive BALB/c mice and even at 50 μg doses there were no toxic effects observed up to 15 hours post-administration of toxins with only 10% of mice showing some toxicity at 24 hrs. We were interested in biodistribution of toxins during the early time points on which the information in literature is lacking.

Intravenous administration of labeled toxin revealed predominant liver and spleen localization as early as 1 and 3 h with significant clearance through the kidneys as manifested by very high uptake in the bladder (Fig. 3a–e). The observation of renal clearance for toxins that are > 80 kDa may be explained by enzymatic degradation of toxins in the blood. Moayeri et al. reported that following bolus injection, PA was rapidly cleaved in the blood by protease that was not cell associated [14]. Very little activity was seen in the thoracic area for all three toxins when injected alone or in combination. These results reflect the distribution of labeled toxins, as the diffusion of $^{99\text{m}}\text{Tc}$ label from the toxins would result in the pronounced uptake of free radionuclide in thyroid and stomach, which are target organs for $^{99\text{m}}\text{Tc}$ -pertechnetate [15]. The results of biodistribution performed 24 hr post-injection confirmed the renal clearance of toxin-derived radioactivity as well as the uptake in the liver, spleen and intestines (Fig. 3f). Our results are consistent with a preliminary report of organ localization of ^{125}I -PA which identified liver and spleen followed by the lungs as target organs for IV injected ^{125}I -PA [16]. The finding of renal excretion shortly after injection strongly suggests that toxins are rapidly degraded after intravenous injection and this could contribute to the variability of mouse toxicogenic assays. Co-injection of "cold" PA with $^{99\text{m}}\text{Tc}$ -EF or $^{99\text{m}}\text{Tc}$ -LF in 3:1 molar ratio, a dose chosen to reflect the stoichiometry of toxin binding to the macrophages altered the biodistribution of

both ^{99m}Tc -EF and ^{99m}Tc -LF: at 24 hr post-injection there was significantly more ($P \leq 0.02$) ^{99m}Tc -EF+PA and ^{99m}Tc -LF+PA in the blood, spleen, kidneys and intestines than ^{99m}Tc -EF and ^{99m}Tc -LF, respectively, which is consistent with a preserved ability of labeled toxins to bind to PA. The difference in liver uptake between ^{99m}Tc -EF and ^{99m}Tc -LF and their combination with PA was not statistically significant. Furthermore, our results help to explain the observations made by Moayeri et. al. [3] who injected LeTx systemically into mice and detected liver necrosis by 18–24 hr post-administration of 100 μg of toxins and significant damage to the spleen and intestines at 18 hr and later. Their study also noted a relative absence of damage to the lungs and kidneys. Consistent with this observation we noted very low uptake of the toxins in the thoracic area. The absence of kidney damage despite significant renal and bladder uptake can be explained by clearance of degraded toxin that is unable to mediate tissue damage. Our study showed that anthrax toxins localized at the reported major sites of organ damage shortly after infusion and supports the view that local damage to abdominal organs sets forth a cascade of events that leads to lethal toxicity even after the toxin is largely degraded and cleared.

To confirm the results observed with the ^{99m}Tc -labeled toxins, we repeated the study with ^{123}I labeling. Imaging at 1 and 3 hr post IV injection and the biodistribution at 24 hr (Fig. 4) demonstrated that except for thyroid and salivary glands uptake - the distribution patterns of iodinated toxins were similar to those of ^{99m}Tc -labeled ones with radioactivity concentrated in the liver, spleen and intestines, and being excreted through the kidneys. The absence of any significant lung uptake is apparent as a “gap” in uptake in the chest area on the early images (Fig. 4a–e) and the scintigraphic imaging data was confirmed by biodistribution data at 24 hr (Fig. 4f). We also noted considerable uptake in the thyroid and salivary glands (organs expressing sodium iodide symporter) for all preparations which is due to dehalogenation – an observation consistent with the reported biodistribution results in [16]. For that reason, administration of any radioiodinated molecule to patients is accompanied by blocking their thyroids with Lugol to prevent radioiodine uptake. Interestingly, in spite of relative instability of iodine label, iodination is still considered a useful method of radiolabeling.

In conclusion, the availability of ^{188}Re and ^{99m}Tc -labeled PA, LF and EF toxins allowed us to confirm the number of PA binding sites per cell, to provide an estimate of the dissociation constant of PA for its receptor, and to demonstrate tissue distribution of toxins after intravenous injection. Also, since imaging is performed at toxin concentration that below pharmacologically significant, these observations revealed metabolism patterns that are not masked by toxic effects.

ACKNOWLEDGEMENTS

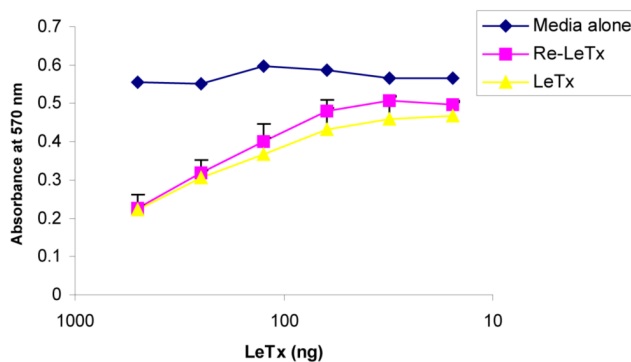
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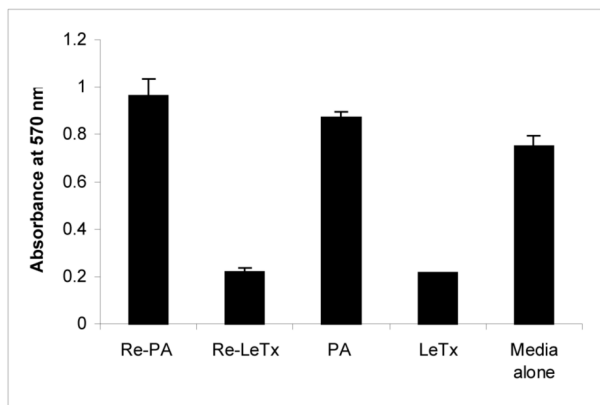
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a)



b)



c)

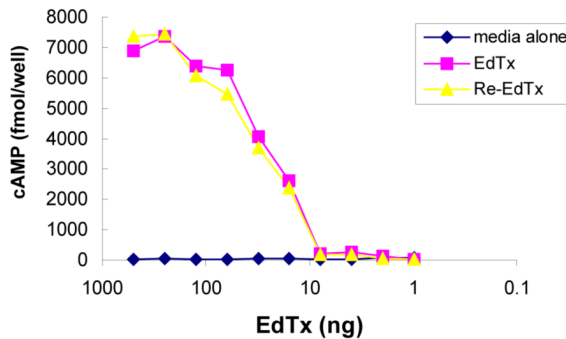


Fig. 1. Evaluation of biological activity of ^{188}Re -labeled lethal toxin and edema toxin in toxicity assays. Lethal toxin (LeTx) is a mixture of PA and LF. ^{188}Re -labeled lethal toxin (^{188}Re -LeTx) is a mixture of ^{188}Re -PA and LF. Edema toxin (EdTx) is a mixture of PA and EF. ^{188}Re -labeled edema toxin (^{188}Re -EdTx) is a mixture of PA and ^{188}Re -EF. a) dose response experiment showing MTT assay of J774.16 macrophage-like cells treated with the increasing doses of LeTx or ^{188}Re -LeTx; b) investigation of possible influence of ^{188}Re radiation on J774.16 macrophage-like cells in the MTT assay; c) cAMP assay of CHO cells treated with EdTx or ^{188}Re -EdTx. The values shown are the averages of 3 measurements and the brackets denote the standard deviation of the measurement.

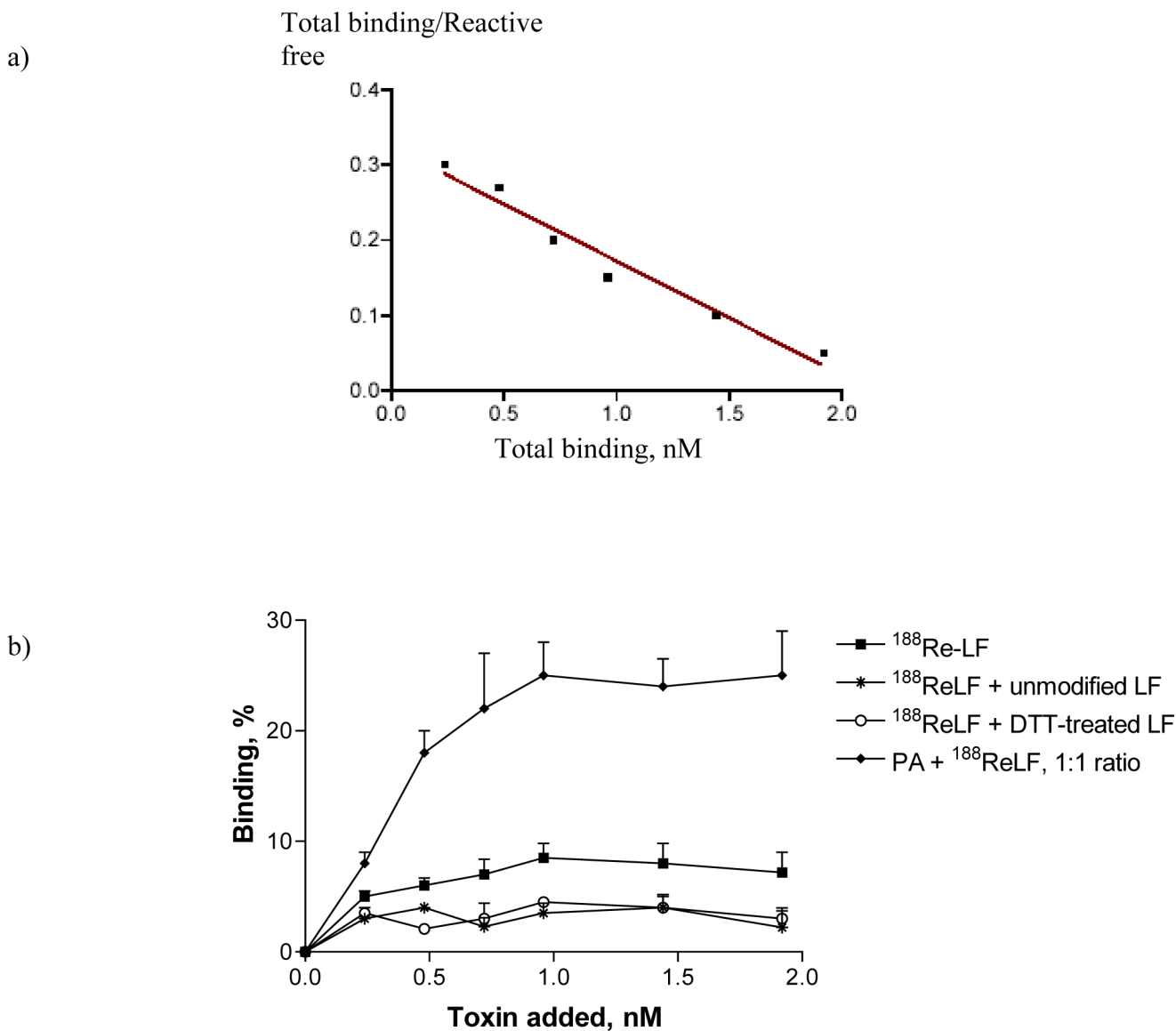


Fig. 2. Binding of ^{188}Re -labeled toxins to macrophages: a) Scatchard transformation of $^{188}\text{Re-PA}$ binding to macrophages; b) binding of $^{188}\text{Re-LF}$ to normal macrophages and to macrophage-bound PA with or without large molar excess of unmodified or DTT-treated LF. The percentage binding on Y axis shows the percentage of total $^{188}\text{Re-LF}$ radioactive counts in the sample which are bound to the cells. The values shown in panel b are the average of 3 measurements and the brackets denote the standard deviation of the measurement.

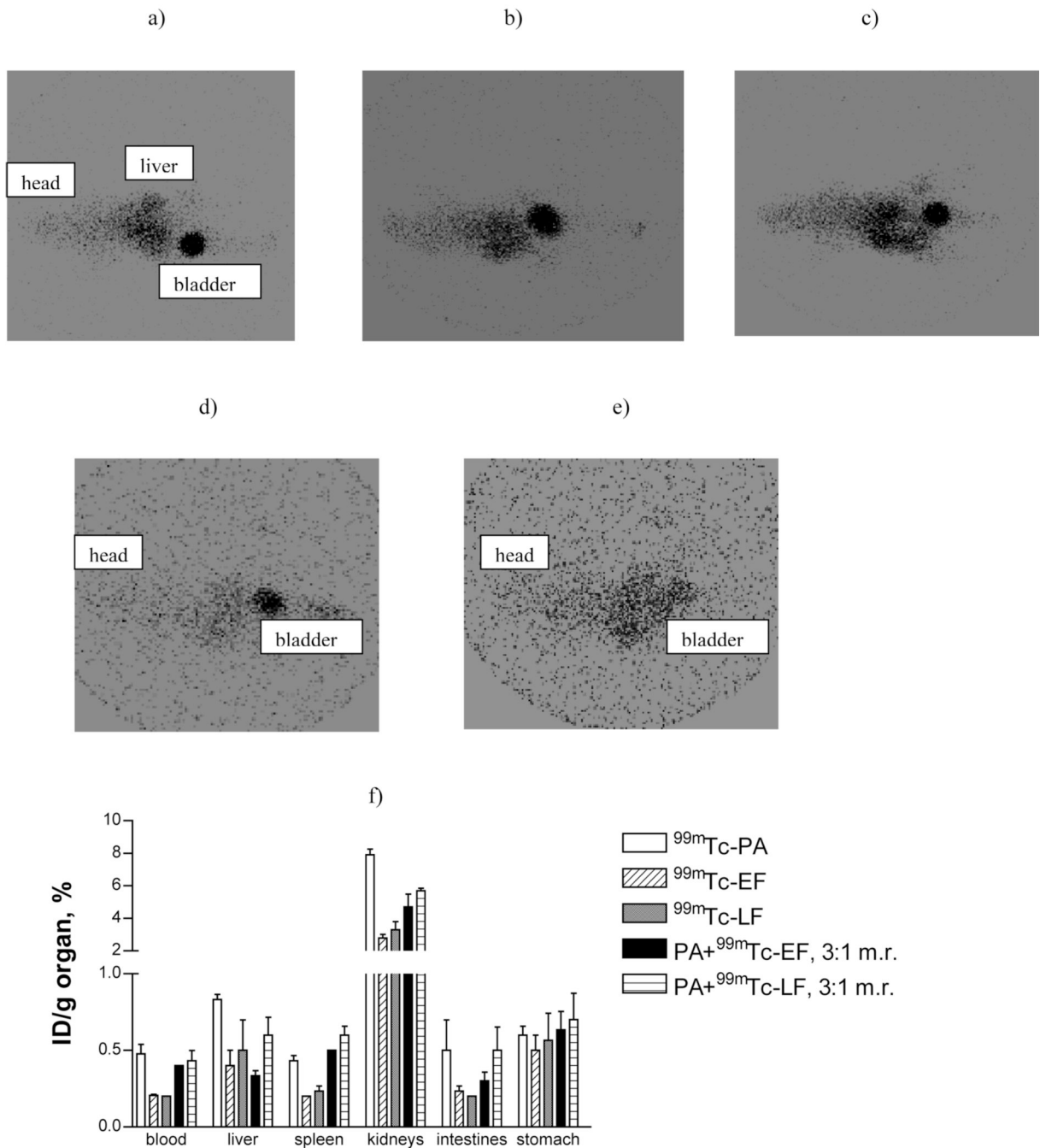


Fig. 3. Scintigraphic imaging and biodistribution of BALB/c mice injected IV with ^{99m}Tc -labeled anthrax toxins. Scintigraphic imaging at 1 hr post-injection (a–e): a) ^{99m}Tc -EF; b) ^{99m}Tc -LF; c) ^{99m}Tc -PA; d) ^{99m}Tc -LF+PA; e) ^{99m}Tc -EF+PA; f) biodistribution at 24 hr. On the images shown all mice are laying on their backs, with the heads on the left and the tails on the right. BALB/c mice were injected IV with: 200 μCi (4.5 μg) ^{99m}Tc -PA; 200 μCi (4.5 μg) ^{99m}Tc -LF; 200 μCi (4.5 μg) ^{99m}Tc -EF; 40 μCi (1.5 μg) ^{99m}Tc -LF co-injected with 4.5 μg "cold" PA; or 40 μCi (1.5 μg) ^{99m}Tc -EF co-injected with 4.5 μg "cold" PA. ID/g - injected dose per gram tissue. Three mice per group were used and the brackets denote the standard deviation of the measurement. M.r. - molar ratio.

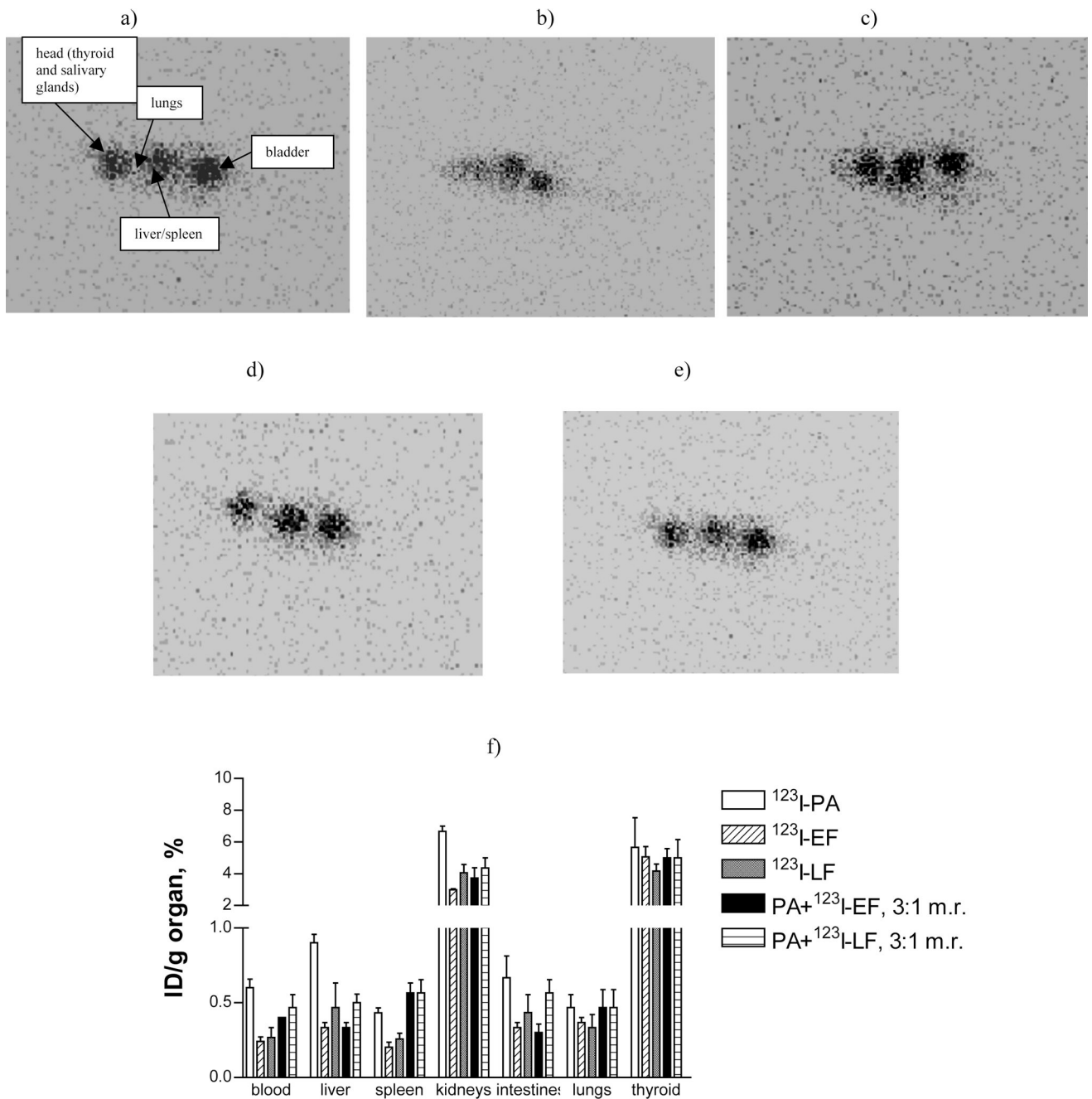


Fig. 4. Scintigraphic imaging and biodistribution of BALB/c mice injected IV with ^{123}I -labeled anthrax toxins. Scintigraphic imaging at 1 hr post-injection (a – e): a) ^{123}I - EF; b) ^{123}I - LF; c) ^{123}I - PA; d) ^{123}I - LF+PA; e) ^{123}I - EF+PA; f) biodistribution at 24 hr. On the images shown all mice are laying on their backs, with the heads on the left and the tails on the right. BALB/c mice were injected IV with: 100 μCi (4.5 μg) ^{123}I -PA; 100 μCi (4.5 μg) ^{123}I -LF; 100 μCi (4.5 μg) ^{123}I -EF; 20 μCi (1.5 μg) ^{123}I - LF co-injected with 4.5 μg "cold" PA; or 20 μCi (1.5 μg) ^{123}I - EF co-injected with 4.5 μg "cold" PA. ID/g - injected dose per gram tissue. Three mice per group were used and the brackets denote the standard deviation of the measurement. M.r. - molar ratio.