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Radiolabeled Antibodies to *Bacillus anthracis* Toxins Are Bactericidal and Partially Therapeutic in Experimental Murine Anthrax▽

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Bacillus anthracis **is a powerful agent for use in biological warfare, and infection with the organism is associated with a high rate of mortality, underscoring the need for additional effective therapies for anthrax. Radioimmunotherapy (RIT) takes advantage of the specificity and affinity of the antigen-antibody interaction to deliver a microbicidal radioactive nuclide to a site of infection. RIT has proven therapeutic in experimental models of viral, bacterial, and fungal infections; but it is not known whether this approach can successfully employ toxin binding monoclonal antibodies (MAbs) for diseases caused by toxigenic bacteria. Indirect immunofluorescence studies with MAbs to protective antigen (MAbs 7.5G γ2b and 10F4 γ1) and lethal factor (MAb 14FA γ2b) revealed the surface expression of toxins on bacterial cells. Scatchard analysis of MAbs revealed high binding constants and numerous binding sites on the bacterial surface. To investigate the microbicidal properties of these MAbs, our group radiolabeled MAbs with either ^{188}Re or ^{213}Bi. In vitro, ^{213}Bi was more efficient than ^{188}Re in mediating microbicidal activity against *B. anthracis*. The administration of MAbs [^{213}Bi]10F4 γ1 and [^{213}Bi]14FA γ2b prolonged the survival of A/JCr mice infected with *B. anthracis* Sterne bacterial cells but not *B. anthracis* Sterne spores. These results indicate that RIT with MAbs that target *B. anthracis* toxin components can be used to treat experimental anthrax infection and suggest that toxigenic bacteria may be targeted with radiolabeled MAbs.**

Radioimmunotherapy (RIT) is a therapeutic method that uses specific monoclonal antibodies (MAbs) labeled with radioisotopes to deliver lethal doses of radionuclides to cells, a technology initially developed for the treatment of cancer (16, 19, 22). Our group recently applied this technique to the treatment of infectious diseases and demonstrated that radiolabeled MAbs to specific pathogens such as *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Streptococcus pneumoniae* can deliver lethal doses of radiation to cells (8, 10, 11). Those studies revealed that specific MAbs labeled with the therapeutic radioisotopes ^{188}Re and ^{213}Bi can reduce the organ microbial burden and prolong survival. Thus, targeted radiation can be used for the therapy of various infectious diseases and has the advantage that it utilizes a relatively small amount of MAb administered after an infection.

Bacillus anthracis is a gram-positive, spore-forming, rod-like bacterium that is the causative agent of anthrax (4). It produces three polypeptides which act in a binary fashion to make up the anthrax toxins: protective antigen (PA), lethal factor (LF), and edema factor (EF) (17, 18). PA, an 83-kDa protein that binds via domain 4 to the anthrax toxin receptor in host cells, is cleaved by a cell-associated furin-like protease into 63- and 20-kDa fragments known as PA_{63} and PA_{20}, respectively. PA_{63} subsequently polymerizes into a heptameric structure that binds to EF or LF and promotes their entry into the cell (17, 18). Edema toxin is a calmodulin-dependent adenylate cyclase which converts intracellular ATP to cyclic AMP, resulting in a significant increase in cyclic AMP levels that culminates in edema (13). Lethal toxin (LeTx) is a zinc metalloprotease that cleaves cellular mitogen-activated protein kinase kinases, which causes the disregulation of the cellular transcriptional machinery and which results in cellular death (13, 14, 17). Hence, toxin production is a critical component of the pathogenesis of *B. anthracis* that results in a wide spectrum of tissue and organ pathophysiologies associated with anthrax (2).

Previously, our group isolated MAbs to the LeTx components of the *B. anthracis* Sterne strain and screened them for LeTx neutralization (20, 21). In this study, we utilized those MAbs to establish that secreted proteins such as bacterial toxins can be targeted by RIT. The results indicate the feasibility of targeting microbes with radiolabeled MAbs to exported proteins, thus significantly increasing the repertoire of potential targets for this therapeutic approach.

MATERIALS AND METHODS

Bacillus anthracis **strains.** *B. anthracis* Sterne strain 34F2 (pXO1 positive, pXO2 negative) was obtained from Alex Hoffmaster at the Centers for Disease Control and Prevention (Atlanta, GA). *B. anthracis* Sterne 34F2 DeltaT (pXO1 negative, pXO2 negative) was obtained from Stephen Leppla at the NIAID (Bethesda, MD). *B. cereus* was obtained from Ernesto Abel-Santos at the University of Nevada (Las Vegas). Bacterial cultures were grown from frozen stocks in brain heart infusion broth (Difco, Detroit, MI) at 37°C for 18 h (mid- to late-logarithmic phase) with shaking. Prior to all experiments with the bacterial strains, the cells were washed and visualized by light microscopy. For spore preparation, Sterne strain 34F2 was grown on sporulating medium agar (Difco, Becton Dickinson, Sparks, MD) for 72 h at 37°C, followed by harvesting in ice-cold water. After three washing steps, the spores were separated from vegetative and partially sporulated forms by centrifugation through a 20 to 50% Histo-Denz gradient. The spore pellet was washed 10 times with water and

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stored at 4°C. Recombinant PA and LF were obtained from Wadsworth Laboratories, New York State Department of Health (Albany).

MAbs. MAbs for PA (MAbs 7.5G γ2b [7.5G] and 10F4 γ1 [10F4]) of *B. anthracis* have been described elsewhere (21). MAb 14FA γ2b (14FA) is specific for the LF of *B. anthracis* and has been shown by in vitro assays to have little neutralization activity (20). MOPC21 immunoglobulin G1 (IgG1; ICN Biomedicals, Aurora, OH) was used in in vitro and in vivo experiments as the irrelevant antibody (Ab) control.

Indirect immunofluorescence. For the surface localization of secreted toxins, *B. anthracis* Sterne 34F2 (10^6) cells were washed with blocking solution (1% bovine serum albumin [BSA], 0.5% horse serum in phosphate-buffered saline [PBS]) and incubated with MAbs for PA (MABs 7.5G and 10F4) and LF (MAb 14FA) at a concentration of 10 μg/ml in blocking solution for 30 min. For these experiments, the bacterial cells were not permeabilized. The cells were again washed with blocking solution and incubated with 10 μg/ml of fluorescein isothiocyanate (FITC)-labeled goat anti-mouse (GAM) IgG (Southern Biotechnology, Birmingham, AL) for 30 min at 37°C in the dark. The cells were washed with blocking solution and suspended in mounting medium (0.1 M *n*-propyl gallate in PBS; Sigma, St. Louis, MO). The slides were viewed with an Olympus IX 70 microscope (Olympus America, Melville, NY) with a ×60 numerical aperture 1.4 optics equipped with standard FITC filters.

Immunoelectron microscopy. Bacterial cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer. For the intracellular localization of the toxins in bacterial cells and spores, sections on nickel grids were etched in a saturated solution of sodium metaperiodate for 60 min, washed with H_2O, and blocked with 1% BSA–0.5% Tween 20 in PBS overnight at 4°C. The grids were incubated with 10 μg of MAbs 7.5G (PA), 10F4 (PA), and 14FA (LF) at room temperature for 2 h. The grids were first washed three times with PBS and then three times with 1% BSA in PBS and were finally incubated with GAM IgG conjugated to 10-nm-diameter gold particles (Goldmark Biologicals, Phillipsburg, NJ) for 2 h. For double labeling, we used GAM IgG conjugated to 15-nm-diameter gold particles (MAb 7.5G, PA) and GAM IgG conjugated to 6 nm-diameter gold particles (MAb 14FA, LF). The samples were then washed with PBS, fixed in 2.5% glutaraldehyde, and then washed with PBS and H_2O. The grids were stained with 4% uranyl acetate and then washed several times with H_2O. The grids were examined with a Jeol 1200 EX transmission electron microscope (Jeol, Peobody, MA).

Radiolabeling of MAbs with ^{188}rhenium and ^{213}bismuth. ^{188}Re (a β-particle emitter) was eluted from an ^{188}Re/^{188}W generator (Oak Ridge National Laboratory, Oak Ridge, TN), while ^{213}Bi (an α-particle emitter) was eluted from a ^{225}Ac generator from the Institute for Transuranium Elements, Karlsruhe, Germany (1a). The MAbs were labeled with ^{188}Re and ^{213}Bi, as described previously (10, 11). Briefly, the MAbs were labeled with ^{188}Re via the reduction of MAb disulfide bonds with dithiothreitol. ^{188}Re was added, and the mixture was incubated for 40 min at 37°C, followed by purification. For labeling with ^{213}Bi, the MAbs were conjugated to a bifunctional chelating agent *N*-[2-amino-3-(*p*-isothiocyanatophenyl) propy1]-*trans*-cyclohexane-1,2-diamine-*N*,*N*′,*N*″,*N*‴,*N*⁗-pentaacetic acid, a kind gift from M. W. Brechbiel (National Cancer Institute, Bethesda, MD), followed by addition of ^{213}Bi for 5 min at 37°C.

Scatchard analysis of MAb binding to *B. anthracis* Sterne 34F2. ^{188}Re-labeled MAbs 7.5G, 10F4, and 14FA were added in 0.12 to 0.60 nM amounts to 10^7 *B. anthracis* Sterne 34F2 cells. After incubation for 1 h at 37°C, the counts in the tubes were obtained with a gamma counter. The cells were then collected by centrifugation and the counts for the pellets were obtained again. Scatchard analysis was used to compute the MAb binding constant and the number of binding sites per cell. The ratio of bound MAb to reactive free MAb was plotted against the specific binding values. The slope and intercept values were obtained via linear regression and were used to calculate the binding constant and the number of binding sites (12). For MAb binding specificity experiments, Scatchard analysis was performed (i) in the presence of excess (10 μg) recombinant PA or LF, in which free toxin was used to compete with MAb binding to bacterial cells, and (ii) with *B. anthracis* Sterne 34F2 DeltaT, which is deficient in toxin and capsule production.

Susceptibility of *B. anthracis* Sterne 34F2 to RIT in vitro. For the ^{188}Re experiments, 10^7 bacterial cells were incubated with [^{188}Re]10F4 (0 to 100 μCi) or matching amounts of unlabeled MAb for 1 h at 37°C with constant shaking. For the ^{213}Bi experiments, a solution of 10^7 bacterial cells was incubated with [^{213}Bi]7.5G, [^{213}Bi]10F4, or [^{213}Bi]14FA (0 to 30 μCi); a matching amount of unlabeled MAbs 7.5G, 10F4, or 14FA; the irrelevant MAb [^{213}Bi]MOPC21 (0 to 30 μCi); or free ^{213}Bi (0 to 30 μCi). The cells were washed, pelleted to remove unbound radioactivity, resuspended in PBS, and incubated at room temperature for 3 h for [^{213}Bi]7.5G, [^{213}Bi]10F4, [^{213}Bi]14FA, and [^{213}Bi]MOPC21 or at 4°C for 48 h for [^{188}Re]10F4. For [^{188}Re]10F4, the 48-h incubation was necessary to allow a longer time for ^{188}Re, which has a physical half-life of 16.9 h, to deliver most of its radiation dose. For ^{213}Bi, a 3-h incubation at room temperature was sufficient, as ^{213}Bi has a short physical half-life of 46 min. To examine the role of the bystander effect, (i) heat-killed *B. anthracis* cells (65°C for 30 min and confirmed by counting of the numbers of CFU) were incubated with radiolabeled MAbs, mixed with live *B. anthracis* cells, and incubated for 4 h without washing; and (ii) *B. anthracis* cells were incubated with 3 μCi of ^{213}Bi. The cells were diluted and plated to determine the numbers of CFU.

MTT cell assay. The 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the toxicity of the toxins to mouse macrophage cell lines. MTT (Sigma) was dissolved at 5 mg/ml in sterile PBS at room temperature, sterilized by passage through a 0.22-μm-pore-size filter, and stored in the dark at 4°C. J774 macrophage-like cells (6×10^4) were incubated in a 96-well plate with 100 ng each of PA and LF and/or 10 μg/ml of MAbs for 4 h at 37°C. The J774 macrophage-like cells (6×10^4) were also incubated only with [^{213}Bi]7.5G, [^{213}Bi]10F4, [^{213}Bi]14FA, or [^{213}Bi]MOPC21 to determine toxicity. A 25-μl volume of a 5-mg/ml stock solution of MTT was added to each well, and after 2 h of incubation at 37°C, 100 μl of the extraction buffer (12.5% sodium dodecyl sulfate, 45% dimethylformamide) was added and cells were then incubated overnight at 37°C. This assay relies on the oxidation of MTT to an insoluble pigment by live cells and is measured by determination of the optical density at 570 nm (Labsystem Multiskan, Franklin, MA).

Treatment of A/JCr mice with radiolabeled MAbs. The efficacy of RIT against *B. anthracis* Sterne infection was tested with A/JCr mice, which are susceptible to bacterial infection. Groups of 5 to 10 female mice were infected intravenously with 10^4 bacterial cells (cells washed and suspended in PBS), which results in the rapid death of the mice. One hour after infection, groups of mice received 100 or 150 μCi of [^{213}Bi]10F4 (PA), [^{213}Bi]7.5G (PA), [^{213}Bi]14FA (LF), or the irrelevant MAb [^{213}Bi]MOPC21 intraperitoneally. The amount of Ab per injection was 15 μg. The mice were monitored daily for mortality and morbidity, and deaths were recorded.

Statistical analysis. Survival was analyzed by log rank analysis with censoring of long-term survival (Sigmastat, Chicago, IL).

RESULTS

Indirect immunofluorescence of *B. anthracis*. We examined the ability of MAbs specific for *B. anthracis* toxin components to bind to bacterial cells. MAbs 7.5G (PA), 10F4 (PA), and 14FA (LF) each bound to bacterial cells in a punctate pattern (Fig. 1). For these experiments, the bacterial cells were not permeabilized, and consequently, Ab binding reflects the surface expression of toxins on germinated bacterial cells. Incubation of *B. anthracis* Sterne DeltaT or *B. cereus* bacterial cells, which do not produce toxin, with MAbs 7.5G, 10F4, and 14FA revealed no binding, confirming the specificities of the MAbs for the toxins (data not shown).

Scatchard analysis of MAb binding to toxins by use of *B. anthracis* Sterne 34F2. We examined the specificities of the MAbs for *B. anthracis* toxin components on bacterial cells by Scatchard analysis. The binding constants of MAbs 7.5G, 10F4, and 14FA were relatively high, with MAb 14FA having an affinity constant that was approximately twofold greater than the constants for MAbs 7.5G and 10F4 (1.16×10^8, 1.0×10^8, and 1.9×10^8, respectively) (Fig. 2A). Additionally, the number of binding sites for MAb 14FA on bacterial cells was approximately twofold lower (1.65×10^3) than the number of binding sites for MAbs 7.5G and 10F4 (2.7×10^3 and 3.1×10^3, respectively). Scatchard analysis of MAb binding with *B. anthracis* Sterne DeltaT revealed no specific Ab binding, and the slopes were not different from zero, according to post-linear regression analysis (Fig. 2B). Addition of exogenous purified soluble PA and LF abrogated the binding of MAbs to bacteria, which confirmed the specificities of the MAbs for the toxins expressed on the surface of germinated bacterial cells (Fig. 2C).

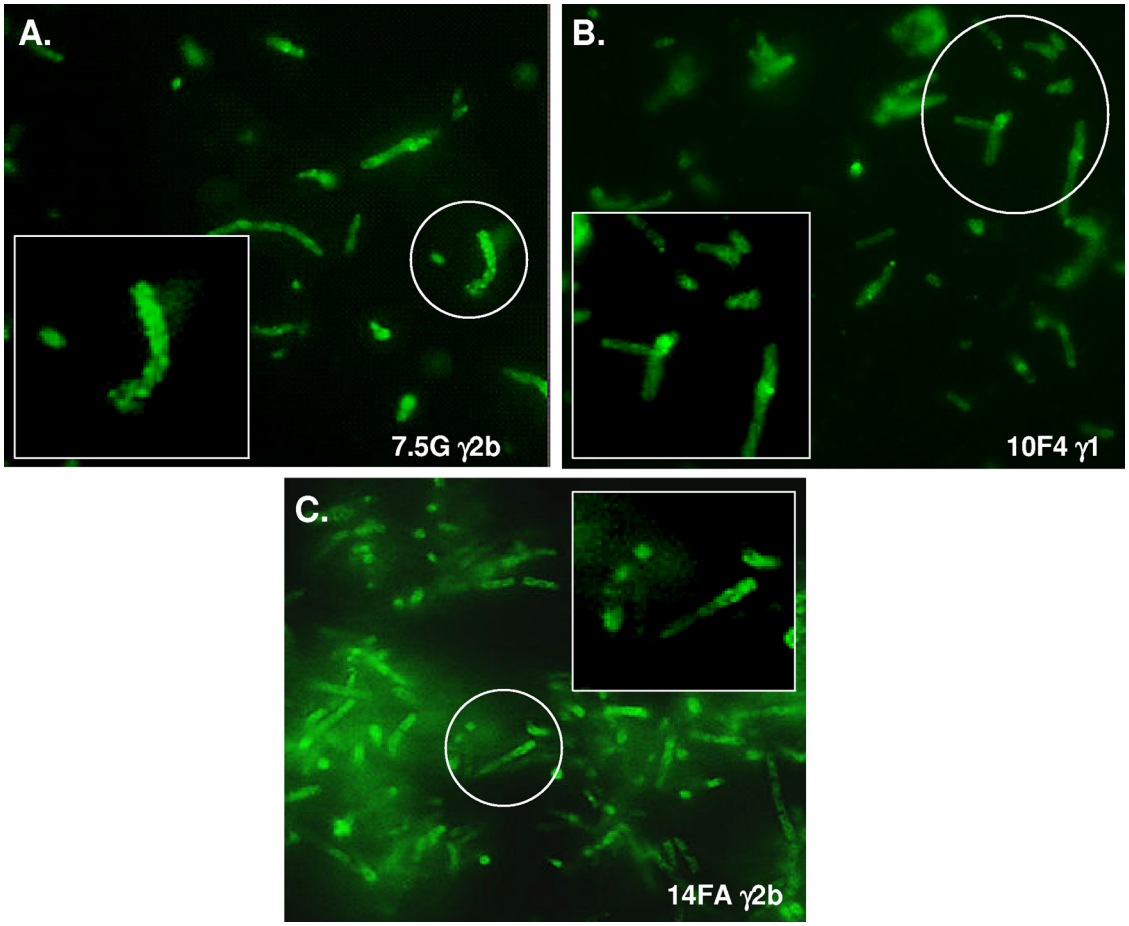

FIG. 1. Indirect immunofluorescence analysis of *B. anthracis* Sterne 34F2. Bacterial cells were not permeabilized for these experiments, and a punctate fluorescence signal denotes binding of the Ab to cell surface-associated toxin. Cells were incubated with MAbs 7.5G (PA) (A), 10F4 (PA) (B), and 14FA (LF) (C). Magnification, ×400. (Insets) Magnifications of individual cells in encircled area.

IEM of MAb binding to *B. anthracis* Sterne 34F2. We used immunogold electron microscopy (IEM) to visualize toxin expression in bacterial cells and spores. IEM of *B. anthracis* spores with MAb 7.5G demonstrated immunogold staining primarily at the spore core, with little or no binding at the exosporium (Fig. 3A). We interpreted this result as indicating that the toxins present in the spores of *B. anthracis* had begun to sporulate and/or that the toxin was being packaged in spores in a preformed fashion for release upon germination. We did not pursue these alternatives but, rather, focused on the loca-

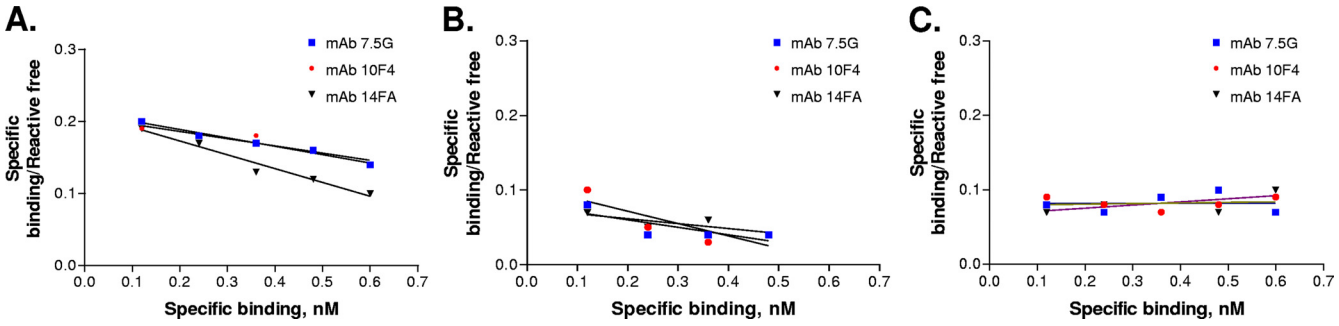

FIG. 2. Scatchard analysis of MAb binding to *B. anthracis* Sterne bacterial cells. (A) Binding of ^{188}Re-labeled MAbs 7.5G (PA), 10F4 (PA), and 14FA (LF) to *B. anthracis* Sterne 34F2 (pOX1 positive, pOX2 negative) bacterial cells to determine the number of binding sites and binding constants. (B) Binding of ^{188}Re-labeled MAbs 7.5G (PA), 10F4 (PA), and 14FA (LF) to *B. anthracis* Sterne DeltaT (pOX1 negative, pOX2 negative) bacterial cells revealed no specific Ab binding, and the slopes were not different from zero. (C) Binding of ^{188}Re-labeled MAbs to *B. anthracis* Sterne 34F2 bacterial cells in the presence of recombinant exogenous PA and LF, which greatly reduced the levels of binding of toxin MAbs.

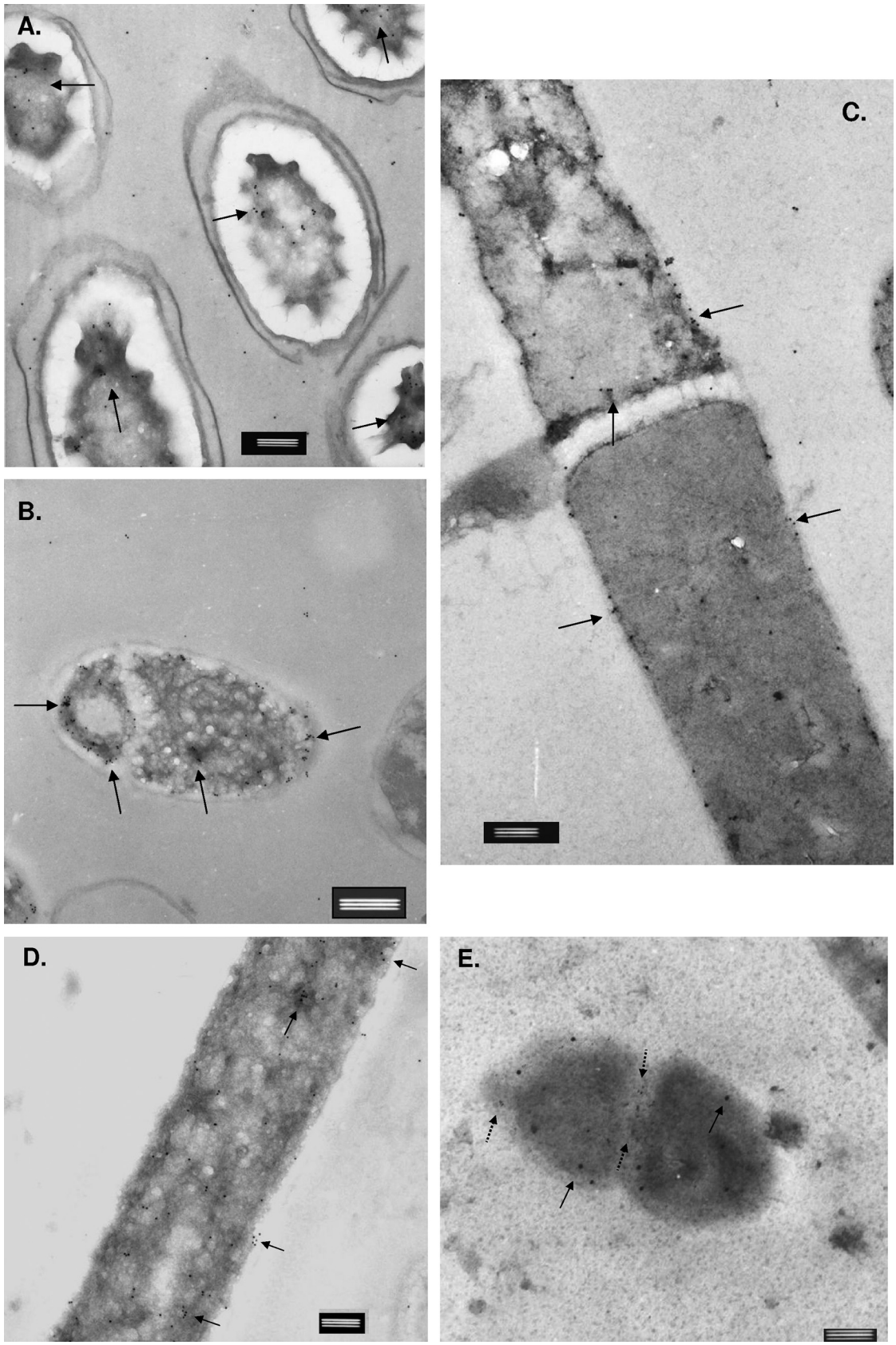

FIG. 3. Immunogold localization of PA and LF in *B. anthracis* Sterne 34F2 cells and spores. (A) Immunogold labeling with MAb 7.5G (PA) revealed gold particles primarily in the core of the spores, with particles occasionally being detected in the exosporium. Immunogold labeling of *B. anthracis* Sterne 34F2 bacterial cells with MAbs 7.5G (PA) (B), 10F4 (PA) (C), and 14FA (LF) (D). Solid arrows, gold particles depicting specific MAb binding. (E) Immunogold double labeling with MAbs 7.5G (PA) and 14FA (LF). Solid arrows, gold particles (15 nm) depicting MAb 7.5G binding; dashed arrows, gold particles (6 nm) depicting MAb 14FA binding. Scale bars, 200 nm.

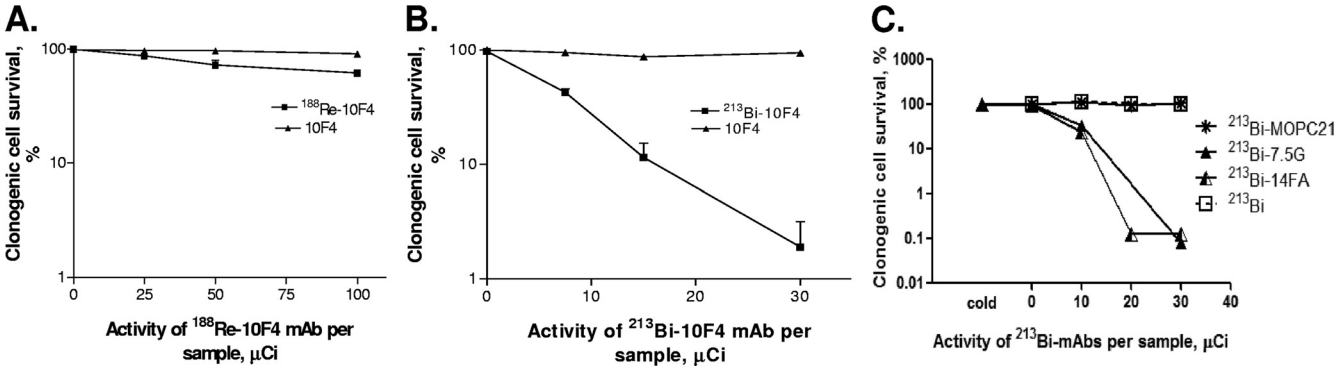

FIG. 4. Susceptibility of *B. anthracis* Sterne 34F2 to radiolabeled MAbs. MAb 10F4 labeled with ^{188}Re (A) and ^{213}Bi (B) was efficient at killing germinated bacterial cells in a dose-dependent fashion. As a control, the cells were treated with matching amounts of unlabeled MAb 10F4: 4.5 to 18 μg (A) and 3 to 12 μg (B). (C) MAbs [^{213}Bi]7.5G and [^{213}Bi]14FA were efficient at killing bacterial cells in a dose-dependent fashion. Free ^{213}Bi and [^{213}Bi]MOPC21 IgG (an irrelevant MAb) had no effect on bacterial cell viability (overlapping curves).

tion of immunogold particles in spores versus germinated cells. When germinated bacterial cells were stained with MAbs 7.5G (PA), 10F4 (PA), and 14FA (LF), immunogold particles were detected at the surface of the bacteria, with staining being observed in the bacterial cytoplasm (Fig. 3B to D). Double labeling of the bacterial cells for PA and LF revealed the binding of gold particles in distinct areas of the bacterial cells but no colocalization of PA and LF (Fig. 3E).

Susceptibility of *B. anthracis* Sterne 34F2 to radiolabeled MAbs. We explored the possibility that radiolabeled MAbs to *B. anthracis* toxins with radionuclides ^{188}Re and ^{213}Bi would convert MAbs with no inherent antibacterial activity into microbicidal MAbs, as we had previously shown for MAbs to *S. pneumoniae*, C. *neoformans*, and *H. capsulatum* (8, 10, 11). Radiolabeling of MAb 10F4 with ^{188}Re conferred some bactericidal activity to an Ig which had no inherent antibacterial activity. Incubation of Sterne 34F2 with 100 μCi of ^{188}Re-labeled MAb 10F4 reduced the numbers of CFU by 25% compared to the numbers of CFU obtained by incubation of Sterne 34F2 with unlabeled MAb 10F4 (Fig. 4A). In contrast, ^{213}Bi-labeled MAb 10F4 was much more efficient at killing bacterial cells than ^{188}Re-labeled MAb 10F4. Incubation of the bacteria with only 30 μCi of [^{213}Bi]7.5G, [^{213}Bi]10F4, or [^{213}Bi]14FA resulted in the complete elimination of bacterial cells in a dose-dependent fashion (Fig. 4B and C). Since the ^{213}Bi-labeled MAbs were more efficacious against bacterial cells than the ^{188}Re-labeled MAbs, additional in vitro studies included only ^{213}Bi-labeled MAbs. ^{213}Bi-labeled MOPC21 IgG (an irrelevant MAb) and free ^{213}Bi had no effect on the viability of bacterial cells (Fig. 4C). In addition, [^{213}Bi]MOPC21, [^{213}Bi]7.5G, [^{213}Bi]10F4, and [^{213}Bi]14FA had no effect on the viability of macrophages, as measured by the MTT assay (Fig. 5A). To investigate whether there was a bystander effect, we determined that 10% of the radiolabeled MAbs (30 μCi) bound to *B. anthracis* cells. Thus, the amount of ^{213}Bi retained after the cells were washed was about 3 μCi. When we incubated bacterial cells with 3 μCi of free ^{213}Bi or with [^{213}Bi]10F4 bound to heat-killed bacterial cells and mixed with live cells, we did not observe any killing of the bacterial cells (Fig. 5B). Thus, bystander killing was minimal to none; and most, if not all, of the bactericidal effects came from the direct killing resulting from the radioactivity emanating from cell-bound radiolabeled antibody. Consequently, we conclude that radiolabeling of PA and LF binding MAbs with ^{213}Bi converted Igs with no inherent antimicrobial activity into microbicidal MAbs.

Survival of A/JCr mice infected and treated with radiolabeled MAbs. Given the greater antibacterial activity of ^{213}Bi

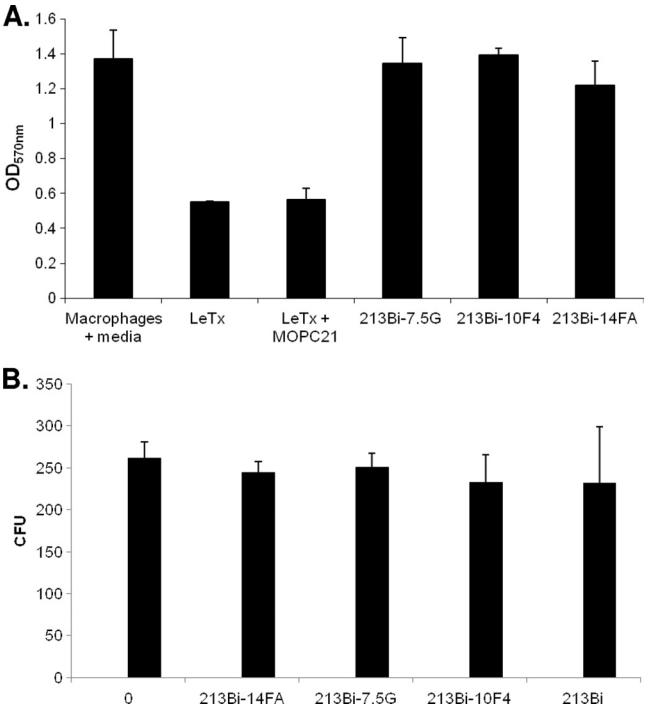

FIG. 5. (A) Susceptibility of J774 macrophages to radiolabeled MAbs. MOPC21 IgG (an irrelevant MAb) did not protect macrophages from the toxicity of LeTx. Incubation of macrophages with [^{213}Bi]7.5G, [^{213}Bi]10F4, and [^{213}Bi]14FA alone had no effect on cell viability by the MTT assay. OD_{570nm}, optical density at 570 nm. (B) Susceptibility of *B. anthracis* Sterne 34F2 to free ^{213}Bi. Addition of 3 μCi of ^{213}Bi to the bacterial suspensions had no effect on bacterial cell viability. The bars represent the averages for four wells per group. Standard deviations are depicted by error bars.

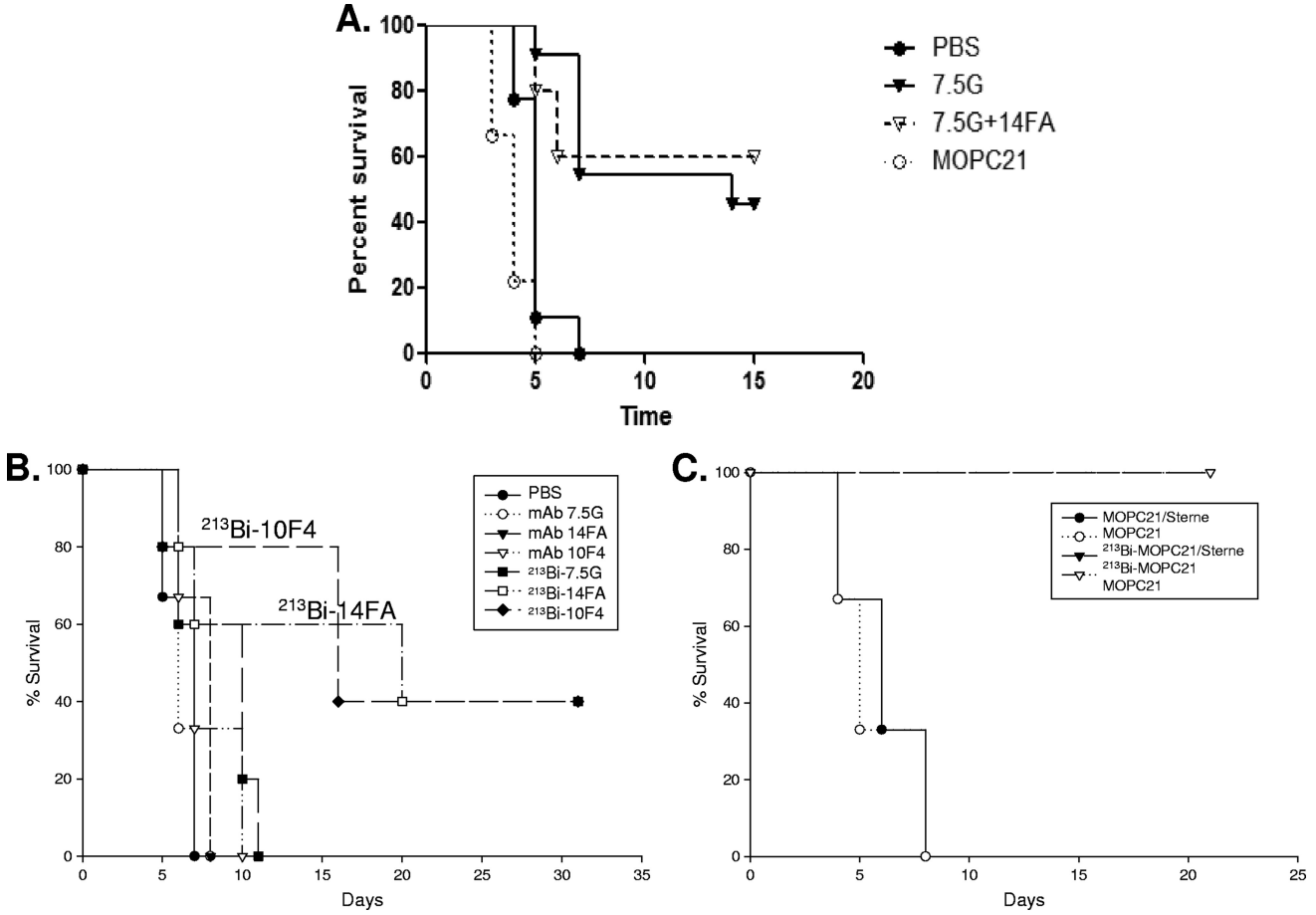

FIG. 6. Studies of survival of A/JCr mice infected with *B. anthracis* Sterne 34F2 in the presence and absence of RIT. (A) Passive administration of MAbs to *B. anthracis* Sterne toxins. Mice were treated with 100 μg of MAb 7.5G or MAbs 7.5G and 14FA and 4 h later were infected with bacterial cells. The mice were treated with MAbs ($n = 10$) or PBS ($n = 10$). (B) RIT of *B. anthracis* Sterne infection with ^{213}Bi-labeled MAbs. The mice were infected 1 h prior to treatment with labeled MAbs. The survival experiment was repeated three times, with similar results being obtained each time. Controls included mice ($n = 5$) receiving unlabeled MAbs in the same amounts (15 μg) as radiolabeled MAbs. (C) [^{213}Bi]MOPC21 and MOPC21 (an irrelevant MAb) did not prolong the survival of A/JCr mice infected with *B. anthracis* Sterne 34F2. [^{213}Bi]MOPC21 and MOPC21 alone had no effect on the viability of A/JCr mice ($n = 5$; overlapping curves).

over that of ^{188}Re, we evaluated the protective efficacy of ^{213}Bi-radiolabeled MAbs 7.5G (PA), 10F4 (PA), and 14FA (LF) in a mouse model of *B. anthracis* infection. MAb 7.5G had previously demonstrated the highest inhibitory activity in vitro for LeTx in a mouse model of toxin injury (21). Since MAbs 10F4 and 14FA exhibited little neutralization activity in vitro (17), we examined only MAb 7.5G in the mouse model of infection. The administration of 100 μg of MAb 7.5G prior to infection prolonged the survival of lethally infected A/JCr mice (Fig. 6A). We proceeded to investigate the therapeutic efficacies of all MAbs when the Abs were radiolabeled and given after infection. In preliminary RIT experiments, mice challenged with *B. anthracis* Sterne spores were given radiolabeled MAbs 7.5G, 10F4, and 14FA; but none of the MAbs prolonged the survival of infected A/JCr mice (data not shown). We attributed this lack of efficacy to the fact that the spores did not express sufficient toxin proteins on their surfaces (23). We subsequently changed the model to infection with germinated *B. anthracis* Sterne 34F2 cells, followed by the administration of radiolabeled MAb 1 h after inoculation. Administration of 100 μCi [^{213}Bi]14FA and [^{213}Bi]10F4 (15 μg) prolonged the survival of mice infected with bacterial cells relative to that of mice receiving PBS and non-isotope-labeled MAbs as controls ($P < 0.05$) (Fig. 6B). The survival experiment was repeated three times, with similar results being obtained each time. There were no differences in the rates of survival of mice treated with 150 μCi ^{213}Bi-labeled MAbs and mice treated with 100 μCi ^{213}Bi-labeled MAbs (data not shown). In addition, the treatment of infected mice with [^{213}Bi]MOPC21 (an irrelevant MAb) had no protective effect on survival, and the administration of radiolabeled Ab did not have any deleterious effects on the survival of uninfected mice (Fig. 6C).

DISCUSSION

In this study, we applied RIT for the treatment of experimental *B. anthracis* infection by targeting bacterial toxins. Previous work had demonstrated that it is possible to direct microbicidal ionizing radiation to microorganisms such as *Cryptococcus neoformans* and *Streptococcus pneumoniae* by la-

beling pathogen-specific MAbs with therapeutic radionuclides such as ^{188}Re and ^{213}Bi (8, 10, 11). However, those studies had used capsule binding MAbs, whereas the approach described here targets a secreted protein, which is presumably only transiently expressed on the bacterial surface.

The application of RIT to bacterial diseases poses different problems than its application to fungal diseases. In contrast to fungal infections, which often take a chronic course, bacterial infections can produce fulminant disease that can damage the host before Ab finds and targets the microbe. Additionally, the difference in sizes between bacterial and fungal cells means that relatively fewer Ab molecules could bind to the bacterial surfaces and that the radiation emitted could fail to damage the microbe by virtue of the fact that bacteria constitute a smaller target. On the other hand, bacteria are potentially more amenable to RIT since they are often more susceptible to external radiation in vitro than fungi (3, 8). Hence, RIT variables that confer efficacy against fungal diseases do not necessarily imply efficacy against bacterial diseases and vice versa.

Initially, we investigated the feasibility of applying the RIT approach to the treatment of experimental anthrax by evaluating the susceptibility of germinated *B. anthracis* Sterne 34F2 cells in vitro. For those studies, we used MAbs which bind to the toxin components of *B. anthracis* Sterne strain 34F2. To that end, our group previously generated and characterized six MAbs to LeTx with significant differences in the ability of those MAbs to neutralize the activities of the LeTx in vitro and in vivo (20, 21). Immunofluorescence studies revealed the binding of MAbs 7.5G (PA), 10F4 (PA), and 14FA (LF) to the surfaces of bacterial cells in a punctate fashion. To our knowledge, the presence of toxins on bacterial surfaces has not been reported before, and surface-attached toxins could represent molecules in the process of secretion. Immunolabeling studies with spores revealed gold particles primarily in the core, consistent with the notion that PA was trapped during sporulation or that there was a low level of PA expression in the spores without export to the surface. We are aware that there are conflicting reports as to whether PA is expressed on spore surfaces, with some reports arguing for such expression (5) and others arguing against it (23). Our immunogold and RIT protection results are most consistent with a paucity of PA in the exosporium, and we interpret the occasional gold particles noted in the exosporium to be consistent with the possibility that spores are beginning germination or that the PA from the original process of sporulation is trapped there.

Scatchard analysis demonstrated high binding constants for the binding of the toxin MAbs to the surfaces of the bacterial cells that were comparable to the binding constants for the binding of the MAbs to surface antigens in *C. neoformans* (6). However, the number of sites for toxin MAb binding to the surfaces of *B. anthracis* cells was almost 500 times lower than the number of sites for MAb binding to the surfaces of *C. neoformans* cells (6). Studies with *C. neoformans* revealed that the efficacy of killing of microbial cells with radiolabeled Abs was directly proportional to the binding constants for the binding of those Abs to their respective antigens (6). In this study, we noted that the radiolabeled MAbs were microbicidal, indicating that the high affinity of toxin MAbs to *B. anthracis* Sterne could compensate for the relatively low number of binding sites on the bacterial surface to ensure that a sufficient amount of labeled Ab would bind to the cell for efficient microbicidal activity. In vitro experiments demonstrated that [^{213}Bi]10F4 (PA) yielded a higher efficiency in killing bacterial cells than [^{188}Re]10F4 (PA). This is because the linear energy transfer of α particles (^{213}Bi) is higher than that of β particles (^{188}Re) (15). In addition, ^{213}Bi has the ability to deliver its radiation dose much faster due to its short physical half-life of 46 min, which matches well with the relatively short doubling times of bacterial species. We therefore utilized toxin MAbs radiolabeled with ^{213}Bi to demonstrate the efficacy of RIT in vivo. In cancer RIT, it has been demonstrated in both preclinical and clinical studies that Abs radiolabeled with α-particle emitters like ^{213}Bi work better against single-cell disease such as leukemia because of their short tissue range, while long-range β-particle emitters such as ^{188}Re perform better in bulky solid tumors because of their greater tissue penetration (for a review, see reference 13). Since bacterial infections most resemble a single-cell disease in cancer, we hypothesized that ^{213}Bi-labeled MAbs might be efficacious in killing bacterial cells. The short physical half-life of ^{213}Bi could also be exploited to facilitate multiple administrations of radiolabeled MAbs (dose fractionation), possibly enhancing efficacy and survival rates.

One of the advantages of RIT is that it uses only a relatively small amount of Ab, thus requiring less protein and circumventing the potential problem of prozone-like effects at high concentrations (6). In our studies, we used an intravenous model of *B. anthracis* infection with intraperitoneal administration of either ^{213}Bi-labeled 10F4 or ^{213}Bi-labeled 14FA, which prolonged the survival of mice injected with *B. anthracis* Sterne strain compared to the length of survival of untreated controls and to the length of survival of mice treated with matching amounts of unlabeled Ab or with irrelevant MAb [^{213}Bi]MOPC21. Since the unlabeled Ab had no therapeutic efficacy, we can attribute the prolongation of survival to the delivery of the radioactive isotope. When the numbers of survivors from all groups in the various independent experiments were tallied, it was apparent that treatment with radiolabeled MAbs was associated with a higher likelihood of surviving the infection, suggesting that in some mice, microbicidal radiation possibly combined with LeTx neutralization from the carrier Ig tipped the scales to enhanced survival. The relative efficacy of the ^{213}Bi-labeled MAbs was 10F4 > 14FA > 7.5G. The greater efficacy of MAb 10F4 relative to that of MAb 7.5 is perplexing, given that both bind to PA with comparable affinities and that unlabeled MAb 7.5G is protective when it is given before infection while unlabeled MAb 10F4 has minimal protective efficacy. Although we do not have a definitive explanation for this finding, we note that MAb 7.5G binds to a linear epitope in domain 1 and mediates protection by inhibiting furin cleavage, while MAb 10F4 binds to a conformational epitope in domain 4 (1, 21). Hence, the difference in efficacy could reflect subtle differences in how these MAbs interact with PA in tissue and/or on *B. anthracis* cell surfaces.

The success of RIT with these MAbs is presumably due to the ability of MAbs to bind to bacteria with a high affinity, despite the low number of binding sites on the surfaces of *B. anthracis* bacterial cells. However, it is also conceivable that some of the efficacy observed derives from binding to soluble PA at sites of infection, resulting in Ab localization sufficiently

near a bacterium to deliver microbicidal radiation. In previous studies, we examined the amount of soluble toxin in infected mice and noted that the amount of PA in the serum is minimal. So, although it is possible that there is some neutralization of circulating toxin by radiolabeled Abs, we believe that the primary mechanism of neutralization comes from the microbicidal activity due to binding to the bacterial surface, since this converts the radiolabeling Ab into a microbicidal molecule and the naked Ab had no efficacy.

The lack of therapeutic efficacy of radiolabeled Ab against spores was puzzling, since we surmised that toxin expression would occur shortly after germination in vivo. Perhaps the lack of efficacy reflects a combination of various factors, including the short time between infection and Ab administration employed, a predominance of germination events within the intracellular spaces of phagocyte cells, and the rapid decay of nuclide activity, all of which combined to negate a therapeutic effect of RIT in spore infection experiments. We note that RIT is designed to combat an infection which is already established in a host. In the case of anthrax, disease occurs after spores have germinated and generated toxins, and consequently, the systemic murine infection model used here is more relevant than a spore infection model to how this therapy might be developed for use by humans. Radiolabeled Abs always exhibit uptake in the liver and spleen and are gradually cleared via the hepatobiliary system (9). Given that the half-lives of murine IgGs are on the order of several days while ^{213}Bi has a very short physical half-life (46 min), ^{213}Bi-labeled Abs would be present in the blood only for about 4 h after administration, with the maximum activity being available only during the first 1 h. After intraperitoneal administration, radiolabeled Abs are present in the bloodstream within approximately 1 h, which should be sufficient for Ab molecules to bind to their respective targets, assuming that they have been accessible. However, if during this time the spores have not germinated or are inside phagocytic cells, radiolabeled Abs will decay and will not be able to target and kill the spores.

In this study, we did not observe that radiolabeled MAbs had toxicity either in vitro, when the macrophages were incubated in the presence of strain Sterne 34F2 with ^{213}Bi-labeled specific MAbs, or in vivo, when healthy mice were given 150 μCi [^{213}Bi]MOPC21, emphasizing the minimal collateral damage to healthy cells from RIT. Previous studies utilizing RIT for the treatment of fungal and bacterial infections examined the hematological toxicity of radiolabeled Abs in mice by obtaining platelet counts (7, 8) and revealed either no drop or only a transient drop in platelet counts which was resolved by day 14 posttreatment. In fact, one of the advantages of using RIT against infections rather than against cancer is that, in contrast to tumor cells, cells expressing microbial antigens are antigenically very different from host tissues and thus provide the potential for specificity and low cross-reactivity with healthy tissues, which results in a low level of toxicity. It should also be noted that the activities of the radiolabeled MAbs administered in our study (150 μCi maximum) are well below the maximum tolerated doses of ^{213}Bi-labeled IgGs which could be administered to mice intraperitoneally (13). In healthy mice, the maximum tolerated dose of ^{213}Bi-labeled IgGs has not been reached, with even doses of greater than over 15,000 μCi producing no toxic effect (12). In infected mice, however, the ceiling for toxicity might be lower because the host is undergoing microbial- and inflammatory-related damage; and in AJ/Cr mice infected with *C. neoformans*, for example, toxicity was observed at 200 μCi (9). The dose-response relationships of RIT for infections are not linear but, rather, have a trapezoidal shape, with the efficacy of treatment rising sharply, reaching a plateau, and then decreasing due to toxicity (this has been discussed elsewhere [12]).

In summary, this study provides a proof of principle for the concept that RIT targeting bacterial toxins can be developed. Although toxins are secreted proteins, they are suitable for RIT because toxin molecules are transiently expressed on the bacterial surface and, consequently, can serve as MAb targets. The findings of our studies suggest that this approach might be applicable to other bacterial toxins and might thus provide a new approach to the MAb-mediated treatment of toxin-based diseases by targeting the microbe through a microbial product. In this study, we used RIT alone as a novel therapeutic method that harnesses the bactericidal power of radiolabeled MAbs to effect a partially therapeutic outcome in experimental *B. anthracis* infection. It is possible that the use of a combination of RIT and antibiotics, vaccines, and the passive administration of cold (unlabeled) Abs targeting different epitopes could be developed to improve clinical outcomes. Although the feasibility of the use of this therapy for human anthrax caused by the inhalation of spores is uncertain, given the complex logistics of RIT, the study shows that radiolabeling with ^{213}Bi converted a MAb with no intrinsic antimicrobial activity into an efficient microbicidal molecule capable of completely eliminating bacterial cells. This observation suggests that Ab modifications that confer microbicidal properties to Igs could provide novel types of therapies against certain types of bacterial pathogens.

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