Specific and Nonspecific Imaging of Localized Fisher Immunotype 1 *Pseudomonas aeruginosa* Infection with Radiolabeled Monoclonal Antibody

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To determine if radiolabeled specific antibodies directed against bacterial antigens could be used to detect sites of infection, gamma camera imaging studies were performed in animals infected with *Pseudomonas aeruginosa*. Murine monoclonal antibodies (Mabs) directed against Fisher Immunotype 1 *Pseudomonas aeruginosa* and a nonmicrobial, nonmammalian haptene, p-arsinilic acid, were labeled with ^{125}I by the Iodogen-Bead method. Unilateral, deep thigh infections were created by innoculation with 2×10^8 Fisher Immunotype 1 *P. aeruginosa*. Twenty-four hours later, one of the radiolabeled antibodies was injected intravenously at a dose of 0.25 mg/kg (100–150 μCi). Serial gamma imaging was then carried out beginning at 4 hr and at ~24-hr intervals thereafter. Beginning as early as 4 hr postinjection, the area of inflammation could be visualized with either the specific or nonspecific Mab, with the images continuing to intensify until 24–48 hr postinjection. At 48 hr, the contrast between lesion and background with the nonspecific Mab began to fade, while the contrast in the specific Mab-generated images continued to intensify until ~192 hr postinjection. Clear-cut differentiation between specific and nonspecific Mab-generated images was possible by 72 hr postinjection. We conclude that specific immune imaging of localized infection with Mab's directed against specific microbial antigens is possible and should be clinically useful. In addition, images created by the localization of immunoglobulin non-specifically at the site of inflammation in the first 24–48 hr postinjection may also provide useful information as to the anatomic location of hidden abscesses.

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Since the original report in 1957 of tumor localization by radiolabeled antibody directed against tumor antigens (*1*), the in vivo delineation of tumor masses by the technique of "specific immune imaging" has received increasing attention (*2*). Antibodies, either polyclonal or monoclonal or their Fab and F(ab')2 fragments, directed against tumor associated antigens (*3–12*) when labeled with either radioiodine or indium-111 (^{111}In), will localize in tumor masses and provide sufficient contrast for external scintigraphy with the conventional gamma camera. In addition to the interest in imaging such tumors, it has been assumed that if tracer quantities of a radionuclide attached to an antibody or an antibody fragment produce a clear image when injected into a cancer patient, then therapeutic amounts of radionuclide or a cytotoxin could also be attached. This would make possible antibody-directed treatment with high local potency but lesser amounts of systemic toxicity (*2*).

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Many of the same clinical issues present in cancer patients are also present in some patients with infection: the need for techniques for anatomically delineating the primary and metastatic sites of infection; the need for noninvasive imaging techniques that could be performed repetitively to assess the response to therapy; the need for a noninvasive technique for in vivo, specific diagnosis that would obviate the need for invasive biopsy procedures; and the need for targeted therapy of such infections as those caused by fungi that would permit more aggressive therapy of the process with lesser amounts of systemic toxicity. It would seem reasonable, then, to apply the experience gained in the specific immune imaging of cancer to the problem of the diagnosis and, perhaps, therapy of localized infection. To explore the hypothesis that specific immune imaging of infectious processes could be accomplished, we have utilized a rat model of Fisher Immunotype 1 *Pseudomonas aeruginosa* soft-tissue infection and radiolabeled specific and nonspecific monoclonal antibodies.

Materials and Methods

Antibodies

A murine monoclonal antibody (Mab) of the IgG_1 subclass specific for an epitope on the O-side chain of *Pseudomonas aeruginosa* Immunotype 1 lipopolysaccharide was utilized as the specific antibody in these studies. This antibody had been produced and characterized previously by one of us (LSY) (*13*). Previous studies had demonstrated that this Mab bound Immunotype 1 lipopolysaccharide in ELISA, immunodiffusion, and immunoblotting assays; agglutinated and opsonized *P. aeruginosa* Immunotype 1 bacteria; and protected against lethal challenge with these organisms in a murine burn infection model. All these properties of the antibody were shown to be immunotype-specific. The antibody utilized in the present studies was affinity-purified from ascites fluid on a protein A column (BioRad Laboratories, Richmond, CA), and the purified material was resuspended in phosphate-buffered saline at a concentration of 1.54 mg/ml (*13*).

Murine monoclonal antibodies directed against the p-arsanilic acid haptene (Ars) were employed as the nonspecific control antibodies in these studies. These antibodies were raised by the immunization of A/J strain mice (Jackson Laboratory, Bar Harbor, ME) with p-arsanilic acid derivatized keyhole limpet hemocyanin (KLH-Ars) according to the regimen of Stahli et al. (*14*). Spleen cells obtained from immune animals were fused with mouse myeloma cell line SP2/O-Ag14 (*15*) at a 5:1 lymphocyte cell ratio using polyethylene glycol (PEG-1000, Baker Chemical Co, Phillipsburg, NJ) as described by Gefter et al. (*16*). Cells were plated in a 96 well microtiter tray and hybridomas selected in medium containing hypoxanthine, aminopterin, and thymidine (*17*). Hybridoma culture supernatants were screened for anti-Ars antibody by enzyme linked immunoabsorbent assay (ELISA) in 96 well polyvinyl chloride microtiter trays coated with Ars-derivatized bovine serum albumin (BSA-Ars) (*18*). Bound antibody was detected by horseradish peroxidase-coated goat anti-mouse immunoglobulin (New England Nuclear Co, Boston, MA). Orthophenylene diamine was used as the substrate, and color development was terminated with 4.5*M* sulfuric acid. Hybridomas were cloned by limiting dilution, and ascites was obtained from pristine primed CAF1 mice injected interperitoneally with 5 million cloned hybridoma cells. Anti-Ars Mab's were purified by affinity chromatography from ascites fluid using BSA-Ars Sepharose columns as described by Lamoyi et al. (*19*) and then reconstituted in phosphate-buffered saline at a concentration of 1.5 mg/ml and frozen in aliquots at −20°C for future use. Hybridoma immunoglobulin subclass determinations were performed by ELISA utilizing reagents purchased commercially (Zymed Laboratories Inc., San Francisco, CA). Anti-Ars antibody 2-12-6 which is of the IgG_1 isotype, and 2-18-11, the $IgG2_b$ isotype, were employed in these studies as nonspecific reagents.

Radiolabeling of Antibodies

Antibodies were radiolabeled with iodine-125 (^{125}I) via the Iodogen Bead method (Du Pont NEN Medical Products, No. Billerica, MA (*20*). Two microliters of iodine-125 were incubated for five minutes in a test tube containing two Iodogen beads. Three microliters of antibody (containing 100–300 μgm of antibody protein in phosphate buffer) was added to the test tube and allowed to incubate for 5 min at room temperature. Saline was added to quench the reaction, and the labeled protein solution was chromatographed on a Sephadex G-25 column to remove unbound iodide. Antibodies were routinely labeled with between 1 and 10 μCi/μg, and prepared for injection. Small aliquots of the antibody were taken before and after labeling and analyzed in the previously described ELISA assays for evidence of possible damage to the antigen-combining site during the labeling process. In no instances could damage be demonstrated; that is, no change in the performance of the antibody in the ELISA assay could be detected after labeling.

Animal Model

A single clinical isolate of *Pseudomonas aeruginosa* Immunotype 1 was utilized to prepare the specific infection model. Control infections were created with clinical isolates of *Pseudomonas aeruginosa* Immunotype 2, *Escherichia coli* and *Staphylococcus aureus*. In each case, the appropriate bacterial strain was incubated overnight on trypticase soy agar plates at 37°C, with individual colonies then picked and diluted with sterile normal saline to produce a turbid suspension containing approximately 2×10^9 organisms/ml. Approximately 200 g, male Sprague-Dawley rats (Charles River Breeding Laboratories, Burlington, MD) were injected into one thigh with 0.1 ml of the suspension which contained ~2×10^8 organisms. Twenty-four hours later, at a time when gross swelling was readily apparent in the thigh, 50 μg of antibody (0.25 mg/kg), labeled with 100–150 μCi of ^{125}I was injected intravenously via the tail vein. As a separate control, a group of animals with infection were injected with 100–150 μCi of free ^{125}I instead of ^{125}I-labeled antibody to determine if free iodine localized at the site of infection. Serial scintigrams were carried out in ketamine anesthetized animals at intervals postinjection utilizing a standard field-of-view scintillation camera (Technicare 420, Solon, OH) with either a pinhole or parallel hole, low-energy collimator. Images were recorded with a peak of 30 keV with a 50% window for a preset time

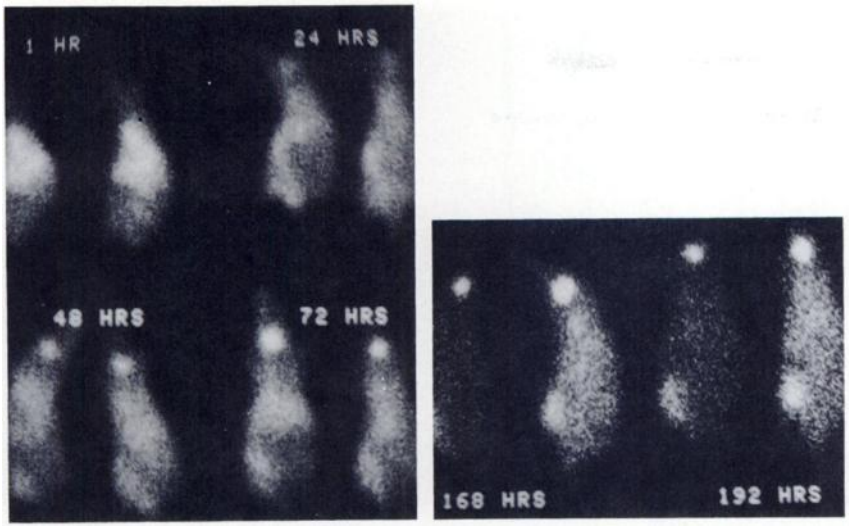

FIGURE 1
Serial scintigrams in animals with immunotype 1 *Pseudomonas aeruginosa* infection of the thigh who have been injected with ^{125}I-labeled monoclonal antibody specific for immunotype 1 *Pseudomonas aeruginosa*. Free iodide is concentrated in the thyroid gland. The same two animals are imaged throughout the 192 hr of the experiment.

of 10 min/view. At the conclusion of imaging, the animals were killed and autopsies performed to evaluate the infection. Cultures of the site of infection and blood were obtained at this time.

RESULTS

A total of 30 animals in seven separate experiments with deep thigh infection due to Fisher Immunotype 1 *Pseudomonas aeruginosa* infection had serial scintigrams carried out with ^{125}I-labeled specific Mab directed against this organism. In all cases, a clearly discernible image of the sites of infection was seen as early as 4 hr postinjection of the antibody, with increasing relative intensity of the lesion observed as long as 192 hr postinjection (Fig. 1). Blood cultures performed on several of these animals were positive for the infecting organism, an observation consistent with the intensity of the local infection (Fig. 2). Cultures from the infected leg were uniformly positive for the infecting strain of *Pseudomonas*.

A total of 20 animals in seven separate experiments with deep thigh infections due to Fisher Immunotype 1 *P. aeruginosa* had serial scintigrams performed after injection with nonspecific Mabs ^{125}I-labeled directed against the p-arsanilic acid haptene. Two anti-Ars Mabs were employed in these studies, an IgG_1, and an $IgG2_b$ antibody. These both produced very similar results: focal uptake was seen in the infected thigh as early as 4 hr postinjection, peak intensity at 24–48 hr, with disappearance by 96 hr (Fig. 3). Clear-cut differences between the images obtained with the specific and the non-specific antibodies were evident by 72 hr and became more marked over the next 72 hr. On occasion, these differences were clearly discernible 48 hr postinjection. Figure 4A is a graphic representation of the ratio of number of counts per pixel of the infected side/number of counts per pixel of the noninfected side in

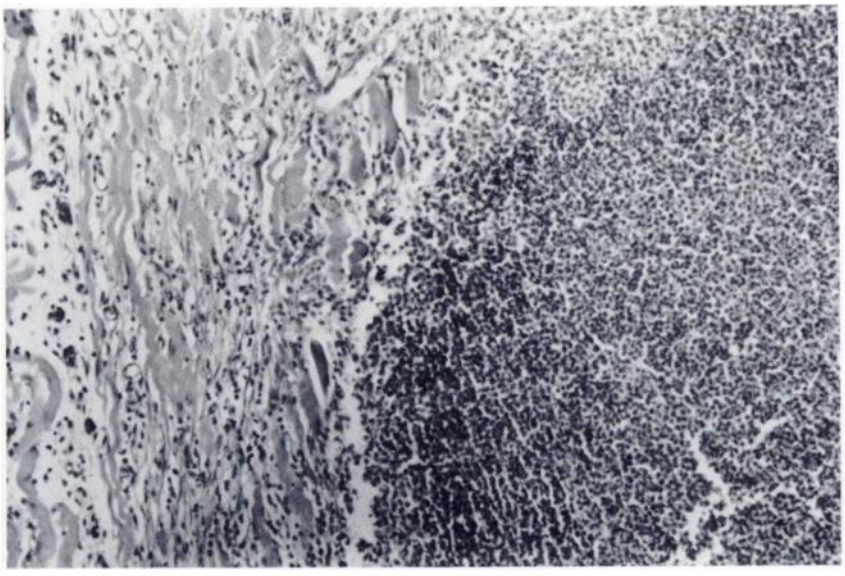

FIGURE 2
Microscopic appearance of inflammatory infiltrate in infected leg of animal inoculated 24 hr previously with 2×10^8 *Pseudomonas aeruginosa*.

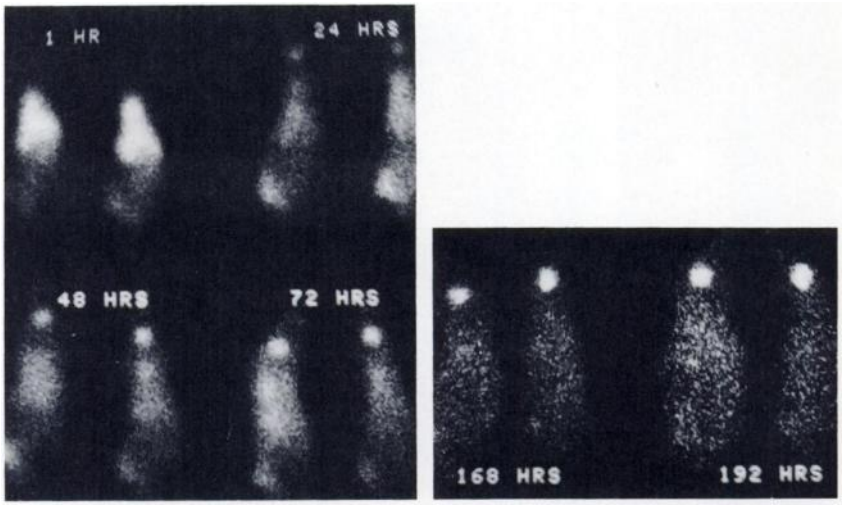

FIGURE 3
Serial scintigrams in animals with immunotype 1 *Pseudomonas aeruginosa* infection of the thigh who have been injected with ^{125}I-labeled nonspecific monoclonal antibodies (an IgG_1 anti-arsenate antibody). The same two animals are imaged throughout the 192 hr of the experiment.

these studies. In Figure 4B, the mean and standard deviations of this ratio in all the experiments is delineated. At 40 hr, there was no significant difference in this ratio for the specific and non-specific antibodies. However, at 100 hr. there was a significantly greater ratio for the specific antibody ($p < 0.02$), and at 144 hr this difference was highly significant ($p < 0.001$).

Animals with infection due to non-Fisher Immunotype 1 *P. aeruginosa* bacteria (Type 2 *P. aeruginosa*, *S. aureus*, or *E. coli*) injected with labeled antibody, either anti-Fisher Immunotype 1 *P. aeruginosa* or anti-Ars, gave similar images over time as those seen with the anti-Ars Ab with Fisher Immunotype 1 *P. aeruginosa* infections (Fig. 3). Three animals scanned 162 hr post-injection (Fig. 5) illustrate the differences between specific and non-specific immunoglobulin concentration in the lesion at this late time following injection. The animal with Fisher Immunotype 1 *P. aeruginosa* infection that was injected with specific antibody has a persistent image; the other two animals, one with staphylococcal infection injected with the anti-Fisher Immunotype 1 *Pseudomonas* antibody, the other with Fisher Immunotype 1 *Pseudomonas* infection injected with the anti-Ars antibody, have minimal retention of antibody at the site of infection.

Neither the iodine-labeled specific nor nonspecific antibodies resulted in opacification of the liver or spleen. Since the animals did not receive any exogenous

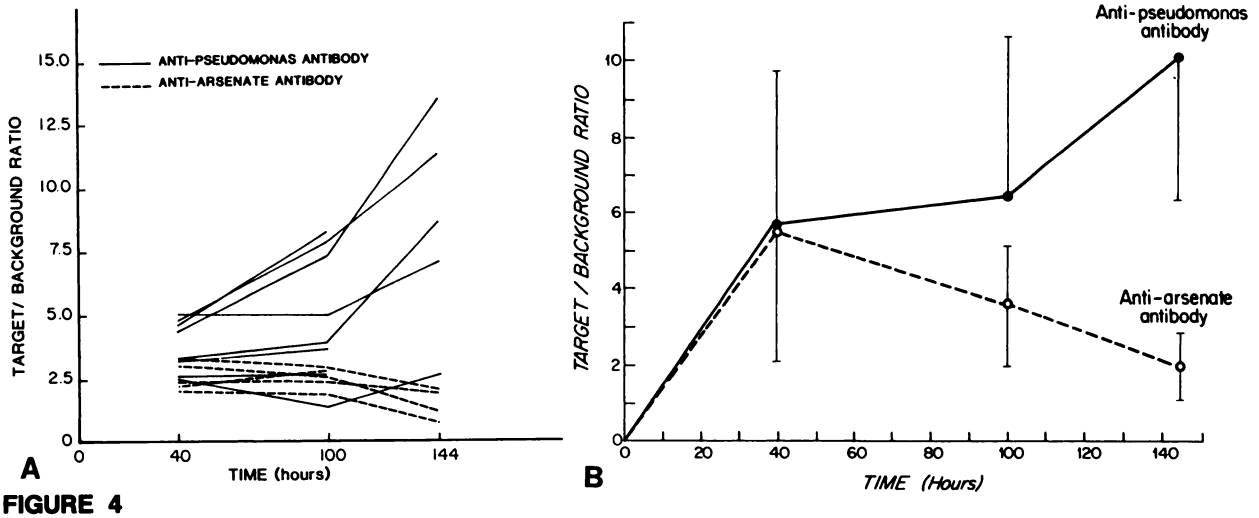

FIGURE 4
A: Graphic representation of the ratio of the number of counts per pixel of the infected side/number of counts per pixel of the noninfected side in a typical experiment, demonstrating the increasing target/background ratio with specific antibody and a decreasing ratio with time when the nonspecific antibody and is employed. B: Graphic representation of the mean and standard deviation of the ratio of the number of counts per pixel of the infected side/number of counts per pixel of the noninfected side in all the experiments performed comparing the images obtained with specific and nonspecific antibody. When the results obtained with the two different antibodies are compared by student's test, there was no significant difference at 40 hr, a significant difference at 100 hr ($p < 0.02$), and a highly significant difference at 144 hr ($p < 0.0001$).

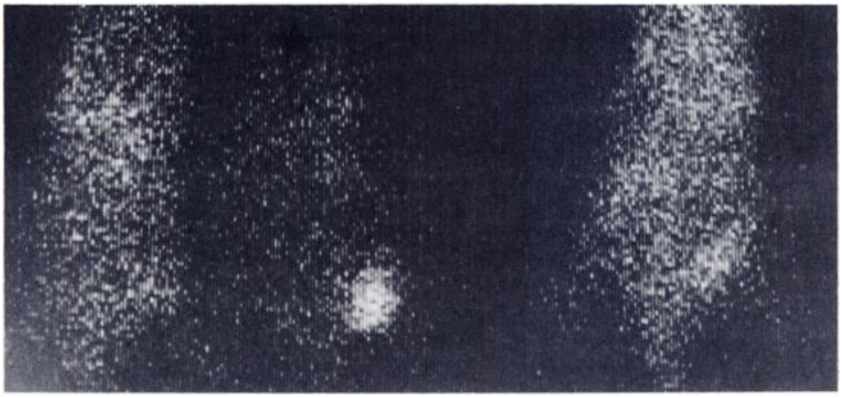

FIGURE 5
The scintigrams 162 hr postinjection of ^{125}I-labeled monoclonal antibody of three different animals are shown. The animal on the left had a chronic thigh infection of 10 days' duration with *Staphylococcus aureus* and had been injected with the anti-*Pseudomonas* antibody; the animal on the right had *Pseudomonas* infection and had been injected with an IgG_{2b} anti-arsenate antibody; the animal in the middle had active *Pseudomonas* infection of 10 days' duration and had been injected with the specific anti-*Pseudomonas* antibody.

"cold" iodine to prevent uptake of the ^{125}I by the thyroid, there is accumulation of the radiolabel over time in the thyroid gland. When animals were injected with free ^{125}I 24 hr following initiation of infection, accumulation of the radiolabel was noted in the thyroid, but there was no accumulation at the site of infection.

DISCUSSION

This study demonstrates that specific immune imaging of a localized site of infection with a radiolabeled Mab directed against a particular microbial antigen is possible, even in gravely ill animals with Pseudomonas bacteremia. Thus, the injection of a Mab at a dose of 0.25 mg/kg will still result in local accumulation at the site of infection despite the potential for antigen-antibody binding within the circulation.

Equally important in these studies is the demonstration that radiolabeled nonspecific Mabs also localized at the site of inflammation at a concentration sufficient to generate a discernible image. Indeed, one can only define specific immune imaging by comparing the images achieved with the specific antibody to those achieved in the two nonspecific situations—a nonspecific antibody (in this case directed against the Ars haptene which is not found on mammalian or bacterial cells) with the same infection; and the putative specific antibody with inflammation due to other organisms. These requirements were fulfilled in these studies, with the interesting observation that for as long as 72 hr postinjection of the ^{125}I-labeled intact antibodies, specific and nonspecific images cannot be distinguished. It is only after this time period that a clear differentiation between specific and nonspecific imaging can be observed.

These observations have led us to propose a model to explain the events that occur following the injection of a radiolabeled Mab in animals (and presumably humans) with localized infection: The first step is the exudation of plasma proteins from the leaking capillary bed at the site of inflammation; this brings the antibody to the vicinity of the infection. The second step is the binding of the specific antibody to the microbial antigens or the nonspecific antibody to nonspecific sites, perhaps Fc receptors on leukocytes at the site of inflammation, thus capturing radiolabeled immunoglobulin at the site of inflammation to an extent sufficient to produce a target:background ratio which is adequate for imaging purposes. The sustained retention of the specific antibody at the site of infection can be explained by the higher affinity of the specific antibody for the microbial antigen than immunoglobulin for Fc receptors, and, perhaps, the rate of turnover of leukocytes at the site of infection. In these studies, it should be emphasized that no computer-based image-enhancement techniques (*21*) or immunoglobulin fragments (*2*, *22*,*23*) were employed. It is likely that one or both of these modifications would greatly accelerate the differentiation of the specific from the nonspecific image.

Based on this model, one might speculate that imaging infectious processes with radiolabeled IgG, either nonspecific or specific, might be easier to accomplish than such imaging of malignant disease. First, the development of an antibody to a microbial antigen not found on mammalian cells should be far simpler than the difficult search for tumor-associated and tumor-specific antigens. Such a specific antibody would have the potential for producing an image with a high target-to-background ratio, which would simplify lesion detection. Second, whereas the microcirculation to tumors and hence the delivery of such blood-borne substances as antibodies can be quite variable, the acute inflammatory process is associated with increased perfusion of the site of interest and an increased egress of circulating proteins from the involved capillary bed. This would optimize the chances for interaction between antigen and antibody, in the case of specific imaging, and between nonspecific IgG molecules and Fc receptors on leukocytes at the site.

The results obtained in this study suggest two parallel pathways to be pursued in developing immunoglobulin-

based imaging techniques for inflammatory processes. The first of these is the possibility of utilizing the nonspecific localization achieved in the first 24–48 hr following injection of the radiolabeled immunoglobulin to find hidden sites of infection. Here, the question is not so much what is causing the infection but rather where is it? An example of the clinical situation in which such an approach might be useful is in the patient with a possible intra-abdominal abscess following trauma or surgery. The microbial etiology is less important than defining the anatomy of the process so that drainage might be accomplished. Thus, nonspecific imaging would be useful.

In contrast, the immunocompromised patient with a localized lesion in the brain or lungs presents a different opportunity. Here, the clinician knows the anatomy but must determine the microbial etiology, which usually requires an invasive diagnostic procedure. In this situation scanning with a radiolabeled antibody specific for *Pneumocystis carinii* or *Aspergillus fumigatus* or *Toxoplasma gondii* could lead to the specific diagnosis without invasion, and also would have the potential of serial studies to monitor the response to therapy.

The results achieved in these pilot studies suggest that both approaches are feasible and should be pursued.

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