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Review Article

Technetium-99m labeled cationic antimicrobial peptides for infection detection and treatment monitoring

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Abstract

Small cationic peptides radiolabeled with technetium-99m are new tools for the scintigraphic imaging of bacterial infections. Due to the specific binding of these peptides to the bacterial membrane, they accumulate at sites of infection but not in sterile inflammatory lesions. This article describes the characteristics of these peptides and the available labeling methods to tag these compounds with technetium-99m, the radionuclide of choice for future human use. Results for the mapping of experimental infections in mice and monitoring the efficacy of antibacterial therapy are also presented. Technetium-99m-labeled cationic antimicrobial peptides, thus, are promising candidates for the scintigraphic visualization of sites of bacterial infection in patients.

Introduction

Febrile patients are sometimes exposed to a large array of diagnostic studies. These include laboratory tests which often reveal nonspecific parameters due to immune host response, such as the erythrocyte sedimentation rate, white blood cell counts and cytokine reactions. For obvious reasons these tests are not specific enough to differentiate between bacterial infection and sterile inflammation. This differentiation is crucial for further clinical analysis and/or treatment. Imaging studies such as X-ray, computed tomography and magnetic resonance imaging show abnormalities caused by morphologic alterations, which may be insufficient to provide a reliable diagnosis. Moreover, such abnormalities can only be detected at advanced stages of disease, so obviously morphologic imaging does not really contribute to an early diagnosis. Conversely, nuclear medicine imaging is based on changes in function, rather than on morphology. In this regard, cationic peptides, belonging to the group of endogenous microbial agents, may be ideal tracers as they have a natural ability to localize at putative sites. Once they are radiolabeled their biodistribution can be visualized and the site of bacterial infection revealed by scintigraphy. This selective behavior is the key to understanding their future role in infection imaging and monitoring of therapeutic response. This paper highlights the position of radiolabeled cationic antimicrobial peptides among the arsenal of less specific agents used for imaging the inflammatory process associated with bacterial infection (1).

Pathophysiology of inflammation

After invasion of a foreign particle such as a microorganism, an inflammatory response takes place which

aims at eliminating the pathogenic insult. In addition, the inflammatory process works to remove the injured tissue and stimulate the regeneration of normal tissue architecture. Once this objective has been reached, specific compounds can be administered to inhibit the chemical mediators which act in a proinflammatory way.

Inflammation is usually characterized as acute or chronic in nature. During acute inflammation, various locoregional events occur which act as a defense and inflammation. They include vascular changes, particularly vasodilatation and increased vascular permeability; the formation of exudate, an extracellular fluid with high protein content and cellular debris; and cellular events such as diapedesis (migration of leukocytes from the smaller blood vessels to the site of tissue injury), chemotaxis and phagocytosis involving the ingestion of foreign particles by polymorphonuclear leukocytes and macrophages.

Infection by pathogenic microorganisms is usually followed by an acute inflammatory response. Tissue macrophages represent the first line of defense. A second line of defense, occurring within hours after the insult, is invasion of the site of infection by large numbers of polymorphonuclear leukocytes. These cells primarily attack by phagocytosis and the engulfed microorganism is exposed to corrosive enzymes, thereby destroying the invader.

Chronic inflammation may be the result of acute inflammation. Under this condition, the number of polymorphonuclear leukocytes is reduced and there is marked proliferation of fibroblasts and infiltration of macrophages, lymphocytes and plasma cells. This third line of defense is active only days or weeks after the initial event. During this late phase, immature monocytes present in blood and bone marrow are transformed into potent phagocytic macrophages. In addition, lymphocytes and plasma cells are recruited, extending the fighting capability.

Radiopharmaceuticals for imaging inflammation/infection

Radiopharmaceuticals are used in medicine as “radioactive tools” for diagnosis and therapy. Radiopharmaceuticals are primarily used in the field of nuclear medicine, in both clinical settings and biomedical research. One of these uses involves the imaging of infection, and over the past decades nuclear medicine has contributed considerably to the management of patients suffering from infectious disease. Early identification and localization of the site of infection is crucial for the appropriate treatment of patients, and in this respect scintigraphy has enjoyed undisputed success. The various radiopharmaceuticals used for these purposes and their localization mechanisms are summarized in Table I.

In clinical practice, however, it is extremely important to discriminate between aseptic inflammation and infection. For example, treating aseptic loosening of a hip prosthesis is quite different from treating an infected prosthesis, although the clinical signs are often similar.

Table I: Radiopharmaceuticals for inflammation/infection imaging.

Migration of white blood cells to infection site
Indium-111 labeled leukocytes
Technetium-99m labeled leukocytes
Increased vascular permeability
Indium-111 and technetium-99m human polyclonal IgG
Gallium-67 citrate
Technetium-99m nanocolloid
Binding to lactoferrin and transferrin at infection site
Gallium-67 citrate
Increased glycolysis of inflammatory cells
Fluoro-18 fluorodeoxyglucose
Binding to white blood cells at infection site
Chemotactic peptides
Interleukins
Binding to bacteria
Gallium-67 citrate
Technetium-99m labeled antimicrobial peptides

Aseptic loosening is caused by an immune response initiated by the phagocytic reaction of the host in an effort to clean the area around the implant from particulate debris produced by component destruction. Aseptic loosening can be observed in approximately 15% of patients within 10 years whereas septic infection may occur in 1-2% of the primary implants (2, 3). Aseptic loosening usually requires a relatively simple revision arthroplasty as opposed to the complex treatment of infection which involves excisional arthroplasty and several weeks of intensive antibiotic therapy followed by a new joint replacement.

In order to be clinically useful, therefore, an imaging test should be able to differentiate between both entities. Moreover, it should be both sensitive and specific. Specificity is particularly important since an unspecific test can easily lead to unnecessary and costly operations and hospitalizations, as well as morbidity and unavoidable mortality associated with surgical interventions. For the sake of completeness, it should be mentioned that the results of joint aspiration have been disappointing and that modern imaging methods like computed tomography and magnetic resonance imaging are hampered by metal-induced artefact. In this context it is clear that the need for developing infection-specific radiopharmaceuticals cannot be overemphasized.

From Table I it can concluded that most of the scintigraphic tests do not allow the distinction between inflammation and infection. Gallium-67 binds not only to bacteria but also to proteins accumulating at sites of both sterile inflammation and bacterial infection. The only tracers which are an exception in this case are the radiolabeled antimicrobial peptides. These agents show selective and specific binding to bacteria and have no affinity to cells associated with the inflammatory response following tissue insult.

Antimicrobial peptides

Peptides are compounds which contain amino acids linked to each other by amine bonds. The term peptide is used when the molecule is comprised of fewer than 100 amino acids (about 10,000 Daltons in molecular weight). Larger peptides are referred to as proteins. When there are fewer than 30 amino acids the term small peptides is used. Proteins and (small) peptides play an essential role in all types of biochemical processes and life does not exist without these compounds.

Antimicrobial peptides play an important role in the immune response after microbial challenge. Their mechanism of action against bacteria is based on the architectural and biochemical composition of the cellular membrane. These differences translate into varying degrees of antimicrobial toxicity and as such they are an important component of the innate immunity against pathogenic infection (4). Antimicrobial peptides display activity against bacteria, virus and fungi *in vitro* (5-7) and against experimented infections *in vivo* (8, 9).

Due to the discovery of antibiotics, the bactericidal effect of various amino acid polymers (10) was ignored. However, interest was restored after the emergence of microorganisms resistant to the most widely used antibiotic agents. These compounds have gained renewed attention as therapeutic drugs after the discovery that antimicrobial peptides are widely distributed throughout the animal and plant world. Cecropin from the pupae of silk moth (11), magainins from *Xenopus* skin (12), defensins from granulocytes (13) and ubiquicidin from the cytosolic fraction of activated macrophages (14) are some examples of these compounds. These peptides have a net positive charge due to an excess of basic residues such as lysine and arginine. They are predominantly produced by phagocytes, epithelial and endothelial cells. This occurs mainly upon contact with microorganisms or microbial products such as lipopolysaccharide and proinflammatory cytokines.

Extensive reviews on the abundance and mechanism of action of these new compounds have recently been published in the scientific literature (4, 15) and is beyond the scope of this article. Briefly, the basis of the antimicrobial activity is the interaction of the cationic (positively charged) domains of the peptide with (negatively charged) surface of microorganisms. The membranes of the latter expose negatively charged lipoteichoic acid and phospholipids, while in mammalian cells negatively charged lipids face the cytoplasm. This difference explains the poor binding of cationic peptides to mammalian cells. The interactions of these peptides with the bacterial cytoplasmic membrane results in permeability ("holes") of the membrane allowing leakage of cellular constituents such as potassium ions, thus destroying the proton gradient across the membrane resulting in bacterial death. Figure 1 is a schematic representation of the role of antimicrobial peptides in the network of innate immunity. It is generally thought that the development of resistance against

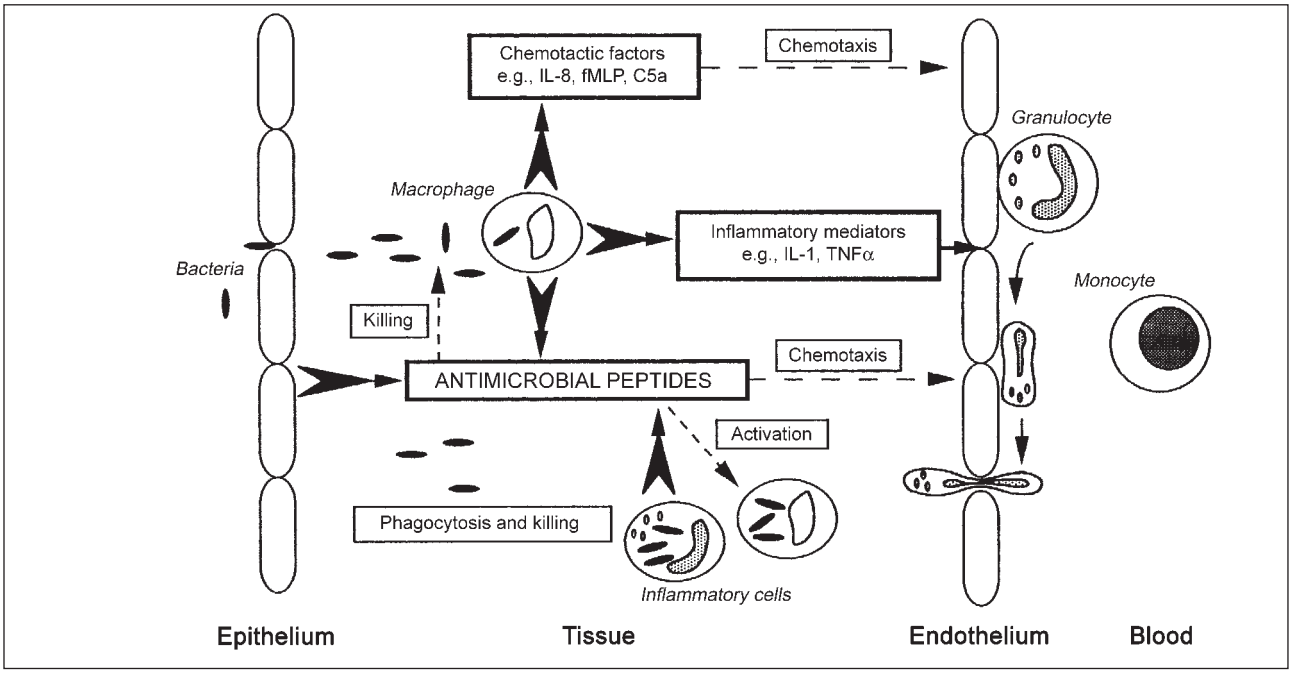

Fig. 1. Antimicrobial peptides in the network of innate immunity presented as a simplified defense system against invading bacteria. Resident macrophages are triggered to release proinflammatory cytokines. These factors induce increased blood flow and increased capillary permeability, the expression of adhesion molecules on endothelial cells and on leukocytes in the circulation, which facilitates the influx of various types of leukocytes. These cells kill bacteria by means of an array of antimicrobial substances including antimicrobial proteins and peptides.

antimicrobial peptides is very limited in nature. This is conceivable as during evolution multiple antimicrobial activities of the host remained active and effective in withstanding invasions by microorganisms. Nevertheless, a mechanism to develop resistance could be the modification of the lipid membrane composition, thereby reducing attractive electrostatic interactions (16).

Fragments of antimicrobial peptides

Peptides may be synthesized using special techniques allowing the production of substantial quantities under good laboratory or manufacturing conditions. This helps to obtain future approval for experimental or clinical use. Moreover, peptide synthesis offers the possibility to study the biological characteristics of peptide fragments, determine active domains and to make chemical variants including, *e.g.*, D-enantiomers, which are less easily degraded by enzymatic decomposition. The study of different domains of native molecules may result in the identification of biologically active regions, *e.g.*, those in antimicrobial cationic peptides which are responsible for binding and killing the target. Eventually, this knowledge and techniques can be used to select peptide fragments that preferentially bind to bacteria and show favorable pharmacologic activity for scintigraphic imaging.

Radiolabeling peptides and peptide fragments with ^{99m}Tc

In general, peptide fragments show rapid pharmacokinetics. They exhibit rapid blood clearance, rapid excretion and, most importantly, rapid penetration of extravascular tissues leading to accumulation in the target. Assuming that they are not degraded by peptides, they are ideal vectors to study various biological phenomena. Their uptake in the target can be visualized using a radiolabeled version of the molecule and with the aid of a gamma camera the pathway of the peptide can be followed over time by scintigraphy. This tool is not only for pharmacologic studies but also plays an important role in clinical patient care.

The most suitable radionuclide for patient studies is technetium-99m (^{99m}Tc) which is readily avaliable at a low cost from a radionuclide generator ("technetium-cow") as ^{99m}Tc-pertechnetate. Moreover, its relatively short half-life of 6 h results in a low radiation burden for the patient and no measurable contamination of the environment. The energy of the photons emitted by this radionuclide is 140 keV, which is ideal for scintigraphic detection with a gamma camera permitting high-quality imaging.

^{99m}Tc should be firmly attached to the peptide and the technetium complex should be sufficiently robust to withstand metabolism itself. ^{99m}Tc-labeling of peptides has been the subject of numerous studies. Technetium complexes in which this element is in oxidation states –1 to +7 are known, although technetium +5 is the most suited oxidation state for incorporation into other molecules. In this

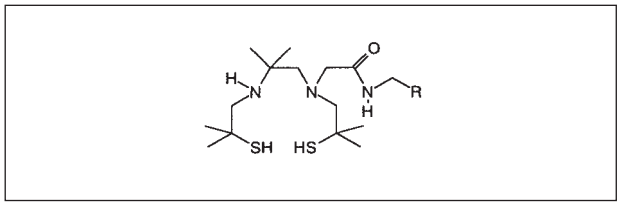

Fig. 2. Bisamine bisthiol for formation of bioconjugate. R = biomolecule.

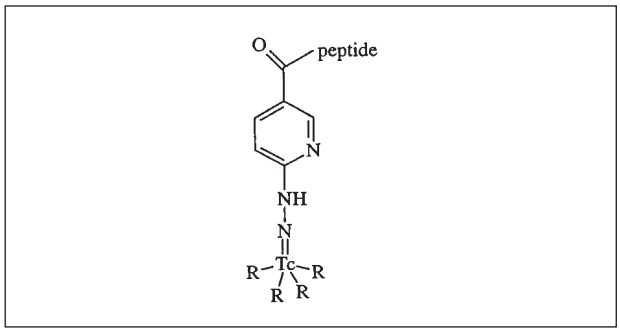

Fig. 3. Structure of HYNIC (hydrazino nicotinamide) peptide complex. R = co-ligands.

+5 state, technetium usually forms stable square pyramidal oxotechnate chelate complex with tetradentate chelating ligands. Donor atoms may be sulfur and nitrogen.

Another feature of this approach is the possibility of incorporating the chelating group for the technetium radionuclide into the peptide during synthesis. This makes it possible to attach this chelating group to the peptide at a site which is remote from the biologically active binding site. In this way, the metal complex should not disturb the biochemical actions of the peptide. Most of these tetradentate ligands contain either one of two thiol groups, with the remainder being *N*-donor atoms as amine or amido groups. They are usually indicated as N_3S or N_2S_2 chelator systems. Particularly, the N_2S_2 (bisamine bisthiol) based chelators have the supposed chemical structure of this technetium complex (Fig. 2).

Another approach is the use of the hydrazino nicotinamide (HYNIC) system developed by Babich *et al.* (17). This conjugate uses a single tether to bind technetium to the peptide thereby offering the possibility to modify the pharmacokinetics of the radiolabeled peptide (Fig. 3). This has been well illustrated by Su *et al.* (18) for the ^{99m}Tc-HYNIC labeling of the small peptide Arg-Gly-Asp.

In contrast to the abovementioned labeling methods, ^{99m}Tc-labeling is also possible using reducing conditions under which the +7 oxidation state in pertechnetate is reduced to the +5 oxidation state. This is problematic for peptides containing intramolecular disulfide bonds which are usually reduced resulting in altered chemical structure and altered biodistribution. Stannous chloride, which is the most commonly used agent to reduce technetium, may reduce such disulfide bonds and is therefore often

Table II: Natural ubiquicidin and synthetic ubiquicidin fragments.

Amino acids	Amino acid sequence	Code
1-59	(14)	UBI 1-59
1-18	KVHGSLARAGKVRGQTPK	UBI 1-18
29-41	TGRAKRRMQYNRR	UBI 29-41
18-29	KVAKQEKKKKKT	UBI 18-29
18-35	KVAKQEKKKKKTGRAKRR	UBI 18-35
31-38	RAKRRMQY	UBI 31-38
22-35	QEKKKKKTGRAKRR	UBI 22-35

unsuitable for ^{99m}Tc binding to peptides, although carefully selected reaction processes may lead to end products with intact biological characteristics (19, 20). This method has important value especially for peptides that do not contain disulfide bonds, although the optimal reaction conditions may often be found only by trial and error. This direct labeling method, without the need of a chelating agent, is a simple, rapid and inexpensive procedure that has been used to label an array of peptides successfully, even those with disulfide bridges, leaving their biological features intact. The mechanism underlying this direct labeling method may involve the reduction of ^{99m}Tc-pertechnetate by stannous chloride in the presence of another reducing agent KBH_4, the production of a TcO intermediate followed by a substitution reaction (20). According to a recent finding by Ferro Flores *et al.* (personal communication), the labeling of a fragment of the cationic antimicrobial peptide ubiquicidin results in a dimeric peptide complex with Arg^3 and Lys being the most suitable binding sites for ^{99m}Tc.

We have used the direct labeling method to label fragment 29-41 of ubiquicidin (UBI 29-41) with ^{99m}Tc. Under acidic conditions (final pH 5-6) and with a relatively short reaction time of 10-20 min a high labeling yield of more than 95% has been found (21), as determined by techniques including high performance liquid chromatography, instant layer chromatography and Sep-Pak analysis. It appeared that the *in vitro* stability of this complex in human serum albumin was excellent (< 15% release of free ^{99m}Tc at 24 h) and that the labeling preparation had retained its biological activity as demonstrated by comparing its *in vitro* bactericidal activity with that of the unlabeled peptide.

Ubiquicidin and ubiquicidin fragments

The growing knowledge about human antimicrobial peptides has motivated us to use synthetic peptides derived from antimicrobial domains of human peptides that bind specifically to microorganisms as radiotracers for infection detection. For this purpose we concentrated our efforts on the human antimicrobial peptide ubiquicidin (6.7 kDa) which was purified from human H292 airway epithelial cells (14). This compound was originally isolated from murine macrophages (22) and in subsequent experiments an identical ubiquicidin was isolated from human cells which allowed us to extrapolate the results obtained in mice to humans. Linear fragments of ubiquicidin were sythesized according to a procedure described elsewhere (23). The amino acid sequences of the ubiquicidin fragments are presented in Table II.

For our experiments we succesfully used the direct labeling technique in order to firmly tag ^{99m}Tc to ubiquicidin fragments. The labeling procedure has been previously described (24).

Identifying ^{99m}Tc-labeled antimicrobial peptides for specific infection detection

Current data on biomolecules provides evidence that biologically potent domains can be identified in antimicrobial proteins and peptides. This opens possibilities for infection detection and promising candidates were selected by us on the basis of a four-phase strategy. First, we tested the *in vitro* binding of the compounds listed in Table II against bacteria and activated human leukocytes. Second, we used a peritoneal infection model to evaluate the peptide binding to bacteria and host cells (24). Third, all peptides were injected into the thigh muscle of animals with an experimental *Staphylococcus aureus* bacterial infection or a sterile inflammatory process created by lipopolysaccharide. From the results of the experiments in these three phases we identified possible candidates for specific infection detection. These candidates showed the highest binding to bacteria and the lowest binding to activated leukocytes in combination with a high *in vivo* accumulation at the infection site and virtually no accumulation at the site of sterile inflammation. In the fourth phase we performed additional experiments on pharmacokinetics and immunological adverse effects. On the basis of this four-phase strategy we chose the ubiquicidin-derived peptide UBI 29-41 as the most promising candidate for specific scintigraphic imaging of infections. Very importantly, in the dose range used in our experiments in mice and rabbits, UBI 29-41 did not exhibit significant antimicrobial activity, thus not destroying its own target. In addition, in the milligram range this peptide was well tolerated by the animals, with no mortality, changes in body weight or alterations in the number of circulating or peritoneal leukocytes observed.

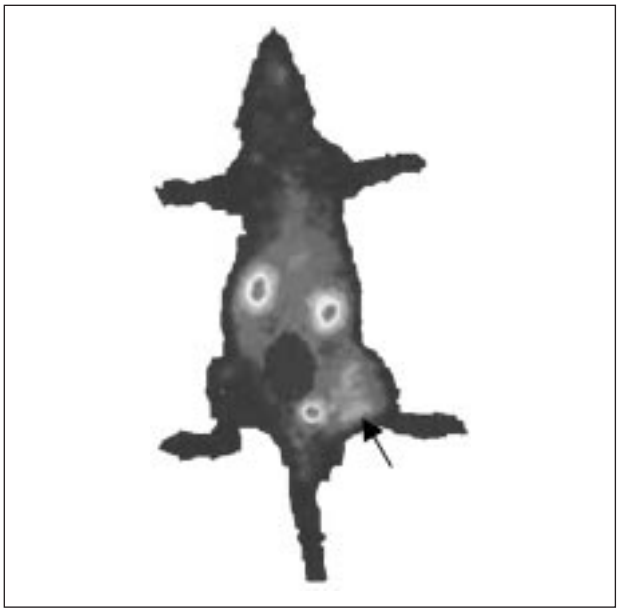

Fig. 4. Scintigram of a rat with a *Pseudomonas aeruginosa* thigh muscle infection (indicated by an arrow) at 2 h after injection of ^{99m}Tc-UBI 29-41.

Scintigraphy with ^{99m}Tc-UBI 29-41

The ability of ^{99m}Tc-labeled UBI 29-41 to discriminate between experimental infections and sterile inflammatory lesions was investigated with scintigraphic studies using immunocompetent mice infected with 10^7 colony forming units (CFU) of bacteria. In these animals sterile inflammation was induced by 100 μg of lipopolysaccharide or large amounts (10^9 CFU) of heat-killed microorganisms. At 18 h postinfection, the ^{99m}Tc-labeled peptide was injected intravenously in a tail vein. The scintigraphic acquisition of the biodistribution took place up to 4 h after injection of the tracer. These studies revealed that the agent accumulates rapidly in the infected tissue and is cleared by the kidneys with no appreciable amount of liver accumulation. An example of a positive scintigraphic image is depicted in Figure 4. In contrast to bacterial infections, ^{99m}Tc-UBI 29-41 did not visualize sterile inflammatory processes.

The accumulation of the tracer, as observed in a scintigraphic image, can be expressed as T/NT (target-to-nontarget) ratios in which T represents the accumulated activity in the infected thigh muscle and NT the activity in the contralateral normal thigh muscle. For ^{99m}Tc-UBI 29-41, this T/NT ratio amounts to about 3 at 1 h after the tracer injection. In the case of sterile inflammation, this T/NT ratio amounts to about 1.2., which is well below the lower limit of detection (1.3). By administering nonradioactive UBI 29-41, the accumulation of radiolabeled peptide could be significantly inhibited. In agreement, the accumulation of ^{99m}Tc-UBI 29-41 at the infection site was higher when a carrier-free preparation was used than when an unpurified agent was used. When using a scrambled version of ^{99m}Tc-UBI 29-41, the accumulation in the infected area was significantly lower. Moreover, the scrambled peptide was unable to decrease the accumulation of the radiolabeled compound, indicating that the amino acid sequence is a crucial feature for binding to bacteria.

In order to evaluate the role of inflammatory cells in the accumulation of ^{99m}Tc-UBI 29-41, a series of experiments with leukopenic mice was carried out. The accumulation of the peptide was not significantly different from that in immunocompetent mice, once more indicating that the binding of ^{99m}Tc-UBI 29-41 to bacteria is the main factor in the visualization of the infection site, rather than binding of the tracer to infiltrating leukocytes.

Monitoring of antimicrobial therapy

In view of the specific binding of ^{99m}Tc-UBI 29-41 to viable bacteria, we considered it useful to investigate the possibility that the radiolabeled peptide could monitor the efficacy of antimicrobial therapy. In this respect we found good correlations between the accumulation of the peptide and the number of viable bacteria. Further experiments were carried out in mice with *S. aureus* infections and administered various doses of antibiotics. The results showed a good correlation ($r^2 = 0.92$, $p < 0.01$) between the accumulation of the tracer in the infected thigh muscle and the antibiotic. This result should be considered in relation to our finding that the target-to-nontarget ratio in erythromycin-treated mice correlated well with the number of viable bacteria ($r^2 = 0.91$, $p < 0.01$) (25). These results, therefore, pave the way for the development of an important clinical tool to monitor antibiotic treatment.

Conclusions

This article describes the usefulness of ^{99m}Tc-labeled cationic antimicrobial peptides for infection detection and monitoring the efficacy of antibacterial therapy in animals by scintigraphy. Furthermore, it was demonstrated that these agents can discriminate between bacterial infection and sterile inflammation. In this respect it was shown that the scintigraphic accumulation is due to binding to viable bacateria rather than association with invading leukocytes. This holds promise for the clinical management of patients in whom this distinction is important, for example in cases of artificial implants for a prosthetic hip or knee.

Our experiments have shown that scintigraphy with ^{99m}Tc-UCB 29-41 may play an important role in these developments. To our knowledge such a diagnostic tool based on a functional abnormality rather than on morphologic alterations has not been available in medicine until now. Obviously, more information on the molecular structure of the radiolabeled peptide and its toxicology is necessary before this agent can be used routinely in patients for infection detection.

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