In Vivo Bioactivity and Biodistribution of Chemotactic Peptide Analogs in Nonhuman Primates

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The dose dependence of the effect of chemotactic peptide on peripheral leukocyte levels was measured in normal Rhesus monkeys. A ^{99m}Tc-labeled hydrazino nicotinamide (HYNIC) derivatized chemotactic peptide analog was used to study biodistribution and inflammation imaging in Rhesus monkeys. In normal animals the studies demonstrated that chemotactic peptide induced a clear dose-dependent reduction in peripheral leukocyte levels. The decrease in leukocyte number occurred almost immediately after injection and rapidly returned to baseline. Significant effects on differential WBC count, blood pressure, pulse rate or respiration rate were not detected. The lowest dose of peptide tested (10 ng/kg) had minimal effect on leukocyte level. The HYNIC derivatized peptide was prepared in excellent yield and purity, had biological activity similar to the native peptide and was readily labeled at specific activity of >20,000 mCi/μmole. When ~0.5 mCi (<2.0 ng/kg) of radiolabeled peptide was injected in monkeys with focal sites of mild sterile inflammation, a pattern of biodistribution similar to radiolabeled WBCs was observed and reductions in leukocyte levels were not detected. At 3 hr after injection, the site of inflammation was readily apparent with a target-to-background ratio of ~3:1. These studies demonstrate that radiolabeled chemotactic peptide analogs are effective agents for imaging sites of inflammation in monkeys. By radiolabeling at high specific activity, the effect of these reagents on peripheral leukocyte levels can be avoided.

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The chemoattractant peptide, N-formyl-methionyl-leucyl-phenylalanine, is a bacterial product that initiates leukocyte chemotaxis by binding to high affinity receptors on the white blood cell membrane (*1–3*). These receptors are present on both PMNs and mononuclear phagocytes. As inflammatory cells respond to the chemoattractant gradient, receptor affinity decreases as additional receptors are expressed, until the cells reach the site of infection, where the concentration of chemoattractant is greatest (*4–6*). Although numerous in vitro structure-activity studies have demonstrated that many synthetic analogs of these small, N-formyl-methionyl peptides bind to neutrophils and macrophages with equal or greater affinity compared to the native peptide (*7–10*), in vivo biodistribution and biological activity studies have been extremely limited.

Recently, we demonstrated that ^{111}In- and ^{99m}Tc-labeled chemotactic peptides are effective agents for the external imaging of focal sites of infection in rats and rabbits (*11,12*). As a prelude to the effective application of these reagents to infection imaging in humans, it is essential to characterize their biodistribution and infection/inflammation imaging properties and biological activity in nonhuman primates. This is particularly important since small doses of these peptides have been shown to produce transient reductions in peripheral leukocyte levels in rabbits (*13*).

In the present study, we report the dose-dependence of peptide-induced reductions in peripheral leukocyte levels in normal Rhesus monkeys. Also, a ^{99m}Tc-labeled hydrazino nicotinamide derivatized chemotactic peptide analog was synthesized and used to study biodistribution and inflammation imaging in monkeys with mild focal sites of inflammation.

MATERIALS AND METHODS

Materials

N-formyl-methionyl-leucyl-phenylalanine (ForMLF), N-formyl-norleucyl-leucyl-phenylalanyl-norleucyl-tyrosyl-lysine (ForNleLFNleYK), phorbol myristate acetate (PMA) and cytochalasin B were obtained from Sigma (St. Louis, MO). ForML[^{3}H]F (60 Ci/mmol) and ^{99m}Tc-pertechnetate (^{99}Mo/^{99m}Tc-generator) were obtained from New England Nuclear (Boston, MA). Hank's balanced salt solution (HBSS) was obtained from Gibco (Grand Island, NY).

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Peptide Synthesis and Characterization

N-Formyl-MLFK and N-Formyl-NleLFNleYK-DTPA were synthesized by standard solid-phase techniques (*14,15*) as previously described (*11*) and purified by high-performance liquid chromatography (HPLC). The nicotinyl hydrazine derivatized chemotactic peptide analog, N-Formyl-MLFK-HYNIC, was prepared as described below.

Succinimidyl-6-t-Boc-hydrazinopyridine-3-carboxylic acid-HCl (171 mg, 0.60 mmole) (*16*) in 1.0 ml of dimethylformamide (DMF) containing 60 μl of diisopropylethylamine was added to a suspension of N-Formyl-MLFK (169 mg, 0.30 mmole) in 2 ml of DMF. The peptide dissolved rapidly and after 2 hr ether-pet ether was added. The upper layer was then discarded and the addition of water to the residue caused a solid to form. The solid was washed with 5% sodium bicarbonate, water and ethyl acetate. The yield of crude product was 183 mg. The protecting group was removed by stirring the crude product with 5 ml of trifluoroacetic acid (TFA) containing 0.1 ml p-cresol for 15 min at 20°C. The TFA was removed by rotary evaporation and ether was added to precipitate the deprotected peptide. The product was purified by reverse-phase HPLC on a 2.5 × 50 cm Whatman ODS-3 column eluted with a gradient of acetonitrile in 0.1% TFA. Fractions containing the major component were combined and the solvent removed to yield the desired product.

The chemical purity of the final product was evaluated by TLC, HPLC, UV spectroscopy, mass spectroscopy and amino acid analysis. Determinations of the EC_{50}s (effective concentration for 50% inhibition) for binding to the chemoattractant receptor on human PMNs and superoxide generation were performed by previously described methods (*11,17,18*).

Radiolabeling with ^{99m}Tc

To avoid the reduction in peripheral leukocyte levels associated with the administration of pharmacologic doses of chemotactic peptide analogs, the HYNIC derivative was radiolabeled with ^{99m}Tc under conditions that maximized specific activity. In this way, with an imaging dose of ^{99m}Tc, the injected mass of peptide will be well below the EC_{50} (~20 n*M*) for receptor activation.

A ^{99}Mo/^{99m}Tc generator was eluted 5 hr after a previous elution to yield a total activity of ~500 mCi. The mass of the Tc and the relative proportion of ^{99}Tc to ^{99m}Tc were calculated by the method of Lamson et al. (*19*). In a typical elution, the total amount of Tc was approximately 3 nmole, the ^{99}Tc-to-^{99m}Tc ratio was ~1.5:1 and the specific activity of the ^{99m}Tc > 100,000 mCi/μmole. Technetium-99m-glucoheptonate (Tc-GH) was prepared from stannous glucoheptonate (Glucoscan, DuPont) to provide the Tc(V) oxo species for radiolabeling the hydrazinonicotinamide conjugated peptide (*15*). Approximately 2.5 ml of ^{99m}Tc-pertechnetate in saline was added to the freeze-dried kit and the final concentration of radioactivity was >150 mCi/ml at the time of preparation. Product radiochemical purity was determined to be >95% by instant thin-layer silica gel chromatography (ITLC-SG) using both acetone and saline as mobile phase solvents.

Approximately 180 μg of HYNIC derivatized chemotactic peptide was dissolved in 50 μl of DMSO and diluted to a final concentration of 10 μg/ml with 0.1 N acetate buffer, pH 5.2. One-half milliliter of peptide solution was placed in a clean glass vial and 0.5 ml of ^{99m}Tc-glucoheptonate was added. The mixture was vortexed briefly and allowed to stand at room temperature for 1 hr. The extent of peptide labeling was monitored by ITLC-SG using three separate solvent systems: acetone, saline and acetone: water (9:1). The ^{99m}Tc-labeled peptide was purified by reverse-phase HPLC on a C18 column (5u, 4.5 × 46 mm, Beckman, Columbia, MD) eluted with a binary gradient. The elution conditions were: solvent A, 5% acetonitrile in 50 m*M* acetate, pH 5.2; solvent B, 50% acetonitrile in 50 m*M* acetate, pH 5.2; gradient: 0% to 100% B over 20 min; flow rate 2 ml/min. The specific activity of the radiolabeled peptide was calculated using the relation: (%RCY × mCi present)/(mmoles of peptide × 100). The solution of radiolabeled peptide was evaporated to dryness under a gentle stream of nitrogen at room temperature. The residue was redissolved in sterile saline and was filtered through a 0.20-micron membrane. Purity of the final solution was evaluated by HPLC using the system described above. Additional details of the radiolabeling procedure were reported previously (*12*).

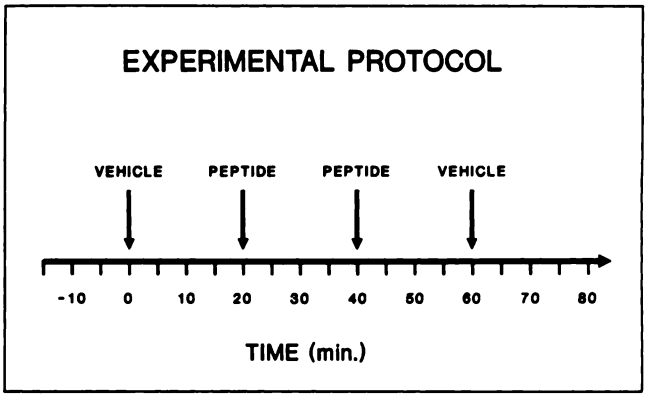

FIGURE 1. Schematic diagram of the experimental protocol. Blood samples were collected for hematological measurements at −15 and −5 min and at 0.25, 0.50, 1, 3, 5, 10 and 20 min after the injections.

Animal Models

Four male Rhesus monkeys, weighing ~10 kg each, were used in these studies. The animals were evaluated with four doses of ForNleLFNleYK-DTPA (10,000, 1,000, 100 and 10 ng/kg) dissolved in 0.2 ml of nonpyrogenic saline. Each animal was treated with all four doses of peptide with a 1-wk resting period between each dose level. The animals were anesthetized with ketamine/xylazine and serial 0.5-ml venous blood samples were collected over 80 min. Each experiment consisted of four injections: vehicle, peptide, peptide, vehicle. Baseline blood samples were drawn at 15 and 5 min prior to the first injection and at 0.25, 0.5, 1, 3, 5, 10 and 20 min after each injection. Immediately after collecting the 20-min blood sample, the next injection was made. Blood pressure, pulse and respiration rate were monitored throughout the experiment. The white blood count (CBC), differential white blood cell count, red blood cell count and packed cell volume were measured on each blood sample. In addition, the animals were monitored for activity and appearance, food consumption and body weight. A schematic summary of the experimental protocol is shown in Figure 1.

Adult female Rhesus monkeys weighing approximately 10 kg were used in the imaging experiments. Since these animals had been used on previous occasions in imaging experiments with other radiopharmaceuticals, they had received numerous intramuscular injections in the right posterior thigh during the induction of anesthesia. These injections resulted in a mild degree of sterile inflammation in some of the animals.

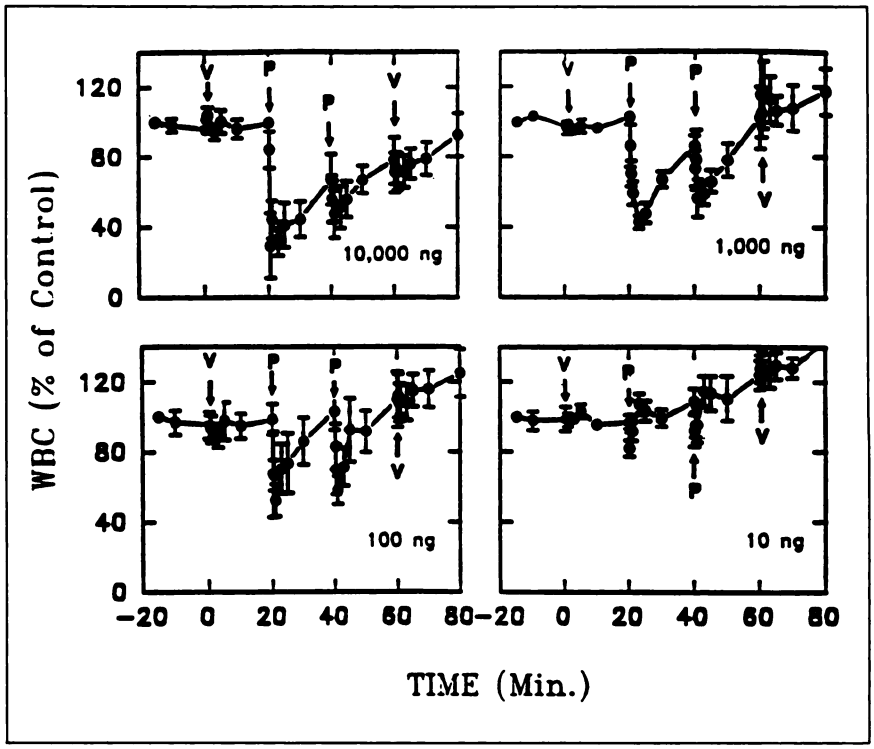

FIGURE 2. Effect of ForNleLFNleYK-DTPA on peripheral WBC levels in monkeys. Total peripheral WBC count is expressed as a percentage of the baseline level. Four doses of peptide (ng/kg) were used. P = peptide injection; V = vehicle injection.

Imaging

Three imaging studies were performed in Rhesus monkeys. Under light ketamine anesthesia (5.5 mg/kg), the animals were injected intravenously through a leg vein with approximately 0.5 mCi ^{99m}Tc-labeled peptide (<2.0 ng/kg). At 5 min, 30 min, 1 hr, 3 hr and 15 hr following injection of radiolabeled peptide, the animals were anesthetized with ketamine/xylazine (15.0 and 1.5 mg/kg) and whole-body scintigrams were acquired using a large field of view gamma camera equipped with a parallel-hole, high-resolution, low-energy collimator interfaced to a dedicated computer system (Technicare Gemini 700, Technicare 560, Solon, OH). The images were acquired at scan rates of 40 cm/min (early images) and 10 cm/min (delayed images), with a 15% window centered on the ^{99m}Tc photo-peak at 140 keV. At 5 min prior to injection and 1, 3, 5 and 15 min after injection, 0.5-ml samples of blood were collected and the CBC was measured. In one of the animals, the blood was centrifuged and percent cell-associated radioactivity was calculated.

RESULTS

The HYNIC derivatized peptide was prepared in excellent yield and chemical purity. The final product showed single bands on TLC and HPLC. UV analysis showed maximum absorption at 268 and 315 nm. Mass spectroscopy gave a m/z at 671. Amino acid analysis was in agreement with the expected product (Met, 1.00; Leu, 1.00; Phe, 0.97; Lys, 1.00). The specific activity of the radiolabeled peptide was >20,000 mCi/μmole after HPLC purification. HPLC analysis of the final injectable solution demonstrated that drying and reconstitution did not cause peptide oxidation or degradation. In vitro assays for binding to the chemoattractant receptor on human PMNs and superoxide generation yielded EC_{50}'s of 2.0 and 20 n*M* respectively.

Figure 2 demonstrates that ForNleLFNleYK-DTPA induces a clear dose-dependent reduction in peripheral leukocyte levels in monkeys. At the two highest doses of peptide (10,000 and 1,000 ng/kg), the leukocyte count decreased to ~40% of the control almost immediately after injection and returned to 60%–80% of control at the time of the second challenge with peptide. After the second injection of peptide, the leukocyte count decreased to 40%–50% of control.

With the highest dose, the leukocyte count returned to 80% of control at the time of the second injection of vehicle and achieved 90% of control at the end of the study, 80 min after the first injection of vehicle. With the 1000 ng/kg dose, the leukocyte count returned to 90% of control at the time of the second injection of vehicle and returned to baseline by the end of the study. After the 100 ng/kg dose, the leukocyte count decreased to ~70% of control almost immediately after injection and returned to baseline by the time of the second peptide challenge. At the time of the second injection of vehicle, the leukocyte level had returned to baseline. With the lowest dose of peptide (10 ng/kg), the decrease in leukocyte count was minimal and returned to baseline within 3 min after each injection of peptide (similar to vehicle injections).

None of the animals demonstrated apparent ill effects after injection of any dose of peptide. Significant effects on differential WBC count, blood pressure pulse rate or respiration rate were not detected.

Figure 3 shows representative, anterior and posterior images of a monkey at 3 and 15 hr after injection of approximately 1.0 mCi of ^{99m}Tc-labeled peptide. In the early

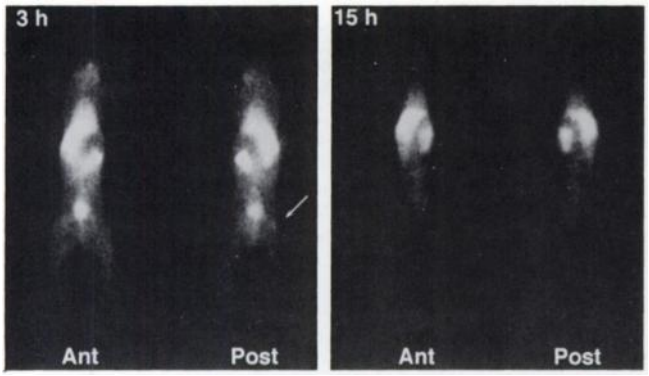

FIGURE 3. Anterior and posterior gamma camera image of a female Rhesus monkey with very mild thigh inflammation (arrow) at 3 and 15 hr after injection of ^{99m}Tc-labeled N-Formyl-MLFK-HYNIC.

image, there were high concentrations of radioactivity in the liver and spleen, consistent with binding to white blood cells. Significant concentrations of radioactivity were also detected in the cardiac blood pool, bone, kidneys and bladder. Lower concentrations were detected in lung, muscle and the GI tract. In addition, the site of inflammation was well visualized at this time (T:B ~ 3:1). At this dose of peptide, there was no significant effect on WBC level. Approximately 25% of the circulating radioactivity was bound to cells.

At the later imaging time, the distribution of radioactivity remained relatively constant. Interestingly, at 15 hr after injection, accumulation of radioactivity at the inflammation site decreased markedly. At both imaging times, a similar pattern of biodistribution was observed in the other animals.

DISCUSSION

The results of this study establish that, as in the rabbit (*12*), chemotactic peptides induce significant transient reductions in peripheral leukocyte levels in monkeys. However, at peptide doses below 10 ng/kg, this effect is not significant. When a ^{99m}Tc-labeled HYNIC derivatized chemotactic peptide was injected in monkeys with mild sterile inflammatory lesions, the distribution of radioactivity closely paralleled the expected pattern of biodistribution for radiolabeled white blood cells. Also, the sites of inflammation were readily detectable within 3 hr after injection with target-to-background ratios of ~3:1. The low level of accumulation of radiolabeled peptide in muscle, lung and the GI tract indicates that it should be useful for imaging infections in these tissues. Even though moderate levels of accumulation were detected in bone and kidney, infections in these organs should also be evaluable. In contrast, accumulations of radioactivity in normal liver and spleen are probably too high for effective imaging of these sites. Since significant concentrations of radioactivity were detected in the kidneys and bladder and low levels were detected in the GI tract, it is likely that renal clearance is the major route of radiopharmaceutical excretion. The observation that only about 25% of the circulating radioactivity is cell-associated suggests that although in vivo leukocyte labeling may be an important mechanism for infection localization, other processes may also contribute. Similar results were obtained in previous studies with rabbits (*12,20*).

By radiolabeling at very high specific activity (>20,000 mCi/μmole), the total mass of peptide in an imaging dose of radiopharmaceutical could be reduced to a level that dose not reduce peripheral leukocyte levels; at 20,000 mCi/μmole, a 0.5-mCi injection in a 10-kg animal corresponds to a peptide dose of <2 ng/kg. Assuming that blood volume is 8% of body weight and a value of 50% for the hematocrit, this dose of peptide corresponds to an initial circulating concentration of ~60 pmole, which is more than two orders of magnitude below the EC_{50} for receptor activation. As expected, this dose of peptide had no effect on peripheral leukocyte levels. Extrapolation of these data to a 70-kg human indicates that an imaging dose of 20 mCi (1.0 nmole) of ^{99m}Tc-labeled peptide should be safe for infection imaging.

These data support the results of previous studies in which we demonstrated that ^{111}In-labeled chemotactic peptides are effective agents for imaging focal sites of bacterial infection in rats (*11*). However, since peripheral levels of rat leukocytes are not affected by these compounds, it was possible that the inflammation imaging properties of this class of molecules could be species-specific. For example, the return of circulating leukocyte levels to normal after a peptide-induced reduction could be due to recruitment of unlabeled cells from bone marrow and not release of radiolabeled cells from sites of sequestration. The results of the present study indicate that this is not the case and establish that radiolabeled chemotactic peptide analogs are effective inflammation imaging agents in animals that are sensitive to their effects on leukocyte levels. Recently, we obtained similar results when the infection imaging characteristics of these peptides were studied in rabbits (*12*) and dogs (unpublished results).

A particularly interesting observation was the fact that although sites of sterile inflammation were highly conspicuous at 3 hr after injection, target-to-background ratios decreased dramatically by 15 hr after injection. This is in marked distinction from our previous results in rabbits and dogs with focal sites of *E. coli* infection, where target-to-background ratios were approximately 3:1 at 3 hr after injection and increased to as high as 20:1 at 12 hr. Possible explanations for this difference include differences in lesion intensity and the difference between bacterial infection and sterile inflammation. If future investigations confirm this difference in lesion kinetics, the utility of the reagents could be significantly increased, since differentiation between infection and sterile inflammation might be possible.

In conclusion, the results of this study establish that the previously reported chemotactic peptide-induced reduction in leukocyte levels in rabbits also occur in monkeys. However, by radiolabeling HYNIC derivatized chemotactic peptides with ^{99m}Tc at very high specific activity, imaging doses of radiopharmaceutical containing concentra-

tions of peptide below the level that reduces peripheral leukocyte levels can be easily prepared. When these formulations are optimized, radiolabeled chemotactic peptides may become safe and effective radiopharmaceuticals for the rapid and accurate identification of focal sites of infection in a variety of clinical situations.

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