ABSTRACT:

Iodinated forms of C-reactive protein (CRP), soluble modified CRP (mCRP-sol), and suspended mCRP (mCRP-susp) were injected iv into CD-1 mice, for analysis of their pharmacokinetics (PK) and biodistribution (BD). The plasma half-life of $^{125}$I-CRP, measured as 4.7 hr, agrees closely with previous reports. The PK and BD characteristics for $^{125}$I-mCRP-sol and $^{125}$I-mCRP-susp were comparable to each other and were distinctly different from those measured for CRP. Whereas $\sim 50\%$ of $^{125}$I-CRP was recoverable from plasma 5 min after injection, only $\sim 5\%$ of $^{125}$I-mCRP was similarly recoverable. The estimated volume of distribution at steady state calculated for either form of $^{125}$I-mCRP was $\sim 10$-fold greater than that calculated for $^{125}$I-CRP ($23.4 \pm 27.6$ and $2.4$ ml, respectively). The estimated mean residence times for $^{125}$I-mCRP were $\sim 2$ times longer than that measured for $^{125}$I-CRP ($9.5 \pm 11.5$ hr, compared with $4.9$ hr). At both 4- and 24-hr time points, substantial amounts of $^{125}$I-mCRP were selectively distributed in the bone marrow. At 24 hr, $\sim 25\%$ of the injected $^{125}$I-mCRP-sol and $^{125}$I-mCRP-susp was localized to the bone marrow (corresponding to $\sim 92\%$ of injected dose/g of tissue). At this time point, only $8\%$ (or $27\%$/g) of $^{125}$I-mCRP was localized to the bone marrow. Overall, the data presented indicate that 1) mCRP has PK and BD characteristics distinct from those of CRP; 2) injected mCRP, although it is rapidly cleared from the general circulation, accesses large body areas and is selectively localized to the bone marrow; and 3) all forms of CRP appear to be excreted in the urine.

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CRP is a major component of the acute-phase response. As part of the immediate defense system of the body, CRP serum concentrations can increase up to 1000-fold within $24 \pm 72$ hr of an infection or tissue insult (Kushner, 1982; Pepys and Baltz, 1983). The in vitro biological activities of CRP include complement activation (Siegel et al., 1974; Kaplan and Volanakis, 1974), opsonization (Nakayama et al., 1982; Kilpatrick and Volanakis, 1991), and activation of macrophages for tumoricidal activity (Zahedi and Mortensen, 1986; Bara et al., 1987, 1988). Both in vitro and in vivo, CRP has been shown to bind to and affect the clearance of nuclear antigens (DuClos, 1996). However, no definitive in vivo function has been identified for CRP.

Our laboratory has established that CRP can exist in a conformationally and antigenically distinct form that we call mCRP (Potempa et al., 1983, 1987). mCRP has been shown to be a naturally occurring protein in various tissues throughout the body (Rees et al., 1988; Egenhofer et al., 1993; Radosavevic et al., 1996). mCRP has immunostimulatory activities that are different from those reported for the native pentameric CRP. These include modulation of leukocyte, monocyte, and platelet activities in vitro (Potempa et al., 1988).

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1 Abbreviations used are: CRP, C-reactive protein; mCRP, modified C-reactive protein; mCRP-sol, soluble modified C-reactive protein; mCRP-susp, suspended modified C-reactive protein; PK, pharmacokinetics; BD, biodistribution; AUMC, area under the first moment curve; MRT, mean residence time; CL, total-body clearance; Vss, volume of distribution at steady state; TCA, trichloroacetic acid.

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both forms of mCRP were then compared with those of the more widely studied CRP molecule.

Materials and Methods

Preparation of Labeled Proteins. CRP was isolated and purified from human ascitic fluids by calcium-dependent phosphorylcholine affinity chromatography, using phosphorylcholine-substituted Bio-Gel resin (Bio-Rad Laboratories, Richmond, CA), and ion exchange chromatography, according to previously published procedures (Potempa et al., 1987). Purified CRP at 1 mg/ml was dialyzed into 25 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 2 mM CaCl₂. CRP was iodinated using Iodo-Bead technology (Pierce Chemical Co., Rockford, IL), as follows. For every 1 ml of CRP solution, six beads were washed with iodination buffer (25 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 5 mM CaCl₂). Na_125I was diluted to 3 mCi/ml of iodination buffer and added to the beads. After 5 min, 1 ml of CRP (at 1 mg/ml) was added and allowed to incubate for 5 min at room temperature. The iodinated protein was removed and passed through a Sephadex G-75 column (Pharmacia, Piscataway, NJ), to separate free label from protein-bound label and to equilibrate the 125I-CRP solution in 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 2 mM CaCl₂.

125I-mCRP-sol was prepared from 125I-CRP by addition of 10 mM EDTA and solid urea truple urea (final concentration, 8 M) and incubation at 37°C for 1–2 hr, followed by dialysis into 10 mM Tris-HCl, pH 8.0 (Potempa et al., 1987). Specific activities for 125I-CRP and 125I-mCRP-sol were calculated based on the total protein concentration of each labeled protein (estimated based on an A_280 value of 1.95 mg/ml) and the 125I activity. An unlabeled sample of mCRP-sol was prepared from CRP by urea chelation/dialysis and was adjusted to 500 µg/ml. Unlabeled mCRP-susp was prepared by incubating CRP at 60°C for 16 hr in the presence of 3 mM EDTA. Unlabeled mCRP-susp was then mixed with 125I-mCRP-sol for the PK/BK study of 125I-mCRP-susp. For each of the protein samples, a stock mixture of labeled and unlabeled protein was prepared, so that each animal would receive a dose of 2.5 mg/kg protein, with 125I activity of 0.5–1.0 × 10⁹ cpm.

Labeled proteins were characterized by UV spectroscopy and radial immunodiffusion. UV wavelength scans obtained for 125I-CRP and 125I-mCRP were identical to those for unlabeled CRP, indicating that protein integrity was maintained during the iodination and conversion processes. Immunodiffusion analysis of radiolabeled CRP confirmed that the protein remained diffusible and retained CRP antigenicity.

Animals. Male CD-1 mice were purchased from Charles River Laboratories, Canada. The mice (age, 35–49 days; weight, 26–31 g) were quarantined for analysis of radiolabeled CRP confirmed that the protein remained diffusable and retained CRP antigenicity.

Whole-blood samples of approximately 300–500 µl were collected from the retroorbital sinus of each animal, under CO₂ anesthesia, into EDTA-containing tubes. Samples were processed to yield plasma and were evaluated for 125I activity in a Packard model 5650 γ-scintillation counter. After determination of plasma 125I activity, an equal volume of 20% TCA was added to each plasma aliquot, to determine the amount of 125I activity that remained associated with intact protein. The samples were briefly vortex-mixed and were placed on ice for 15 min. The aliquots were centrifuged at approximately 3500 g for 10 min, and the supernatant, containing free label or label associated with fragmented protein, was aspirated from each sample. The resultant TCA-precipitated pellet was analyzed for 125I activity. Wherever possible, duplicate samples were processed and the values were averaged.

Urine and feces samples were collected from mice that were individually housed in metabolism cages. Total excreted feces and total voided urine, if present, were collected at 4 or 24 hr. Urine samples collected from each animal were kept separate. The volume of each collected sample was recorded, and, when possible, duplicate 0.05-ml aliquots of each sample were analyzed for 125I activity in a γ-scintillation counter.

Fecal samples were processed and evaluated for 125I activity. Each sample was weighed before homogenization with sufficient water to permit reasonably accurate pipetting. When possible, duplicate 0.2-ml aliquots of each homogenate were analyzed for 125I activity.

After collection of the final blood samples, the animals were anesthetized by injection of sodium pentobarbital. The mice were then injected ip with approximately 1000 units/kg heparin and, immediately before euthanasia, were perfused with a minimum of 1 blood volume of 0.9% saline solution containing heparin. After perfusion and exsanguination, tissues/organs were collected, trimmed of extraneous fat and connective tissue, emptied and cleaned of all contents, and individually weighed before determination of 125I activity. For tissues too large to evaluate in toto, a representative sample of ≤0.5 g was evaluated.

Data Analysis. PK parameters were calculated for each form of CRP by noncompartmental analysis using statistical moment theory (Riegelman and Collier, 1980). The AUC and AUUMC were calculated by the linear trapezoidal rule, using the following equations.

\[
\text{AUC}_{0–t} = (C_0 + C_{\infty})/2(t_0 – t_1) + C/K_e
\]

\[
\text{AUUMC}_{0–t} = \frac{t_0 C_0 + t_1 C_{\infty}}{t_2 - t_1} \times 10^1 + C/K_e
\]

Because of the nature of the plasma data, the elimination rate constant (K_e) could only be estimated as the slope of the line formed by the last two blood collection time points. The estimated MRT was calculated from the AUCM/AUC ratio. The estimated half-life of elimination was calculated as 0.693/K_e. The estimated CL was calculated as the mean dose/AUC ratio. The estimated Vss was calculated as CL × MRT.

Tissue Distribution Analysis. The tissue and organ accumulation of radiolabeled protein in 4 and 24 hr after iv administration was estimated by assuming that the 125I-labeled protein complex was stable. The distribution was expressed either as the percentage of the injected dose recovered in each collected tissue or organ or as the percentage of the injected dose recovered in each tissue or organ divided by the weight of that tissue or organ (in grams). The values therefore have units of percent or percent per gram, respectively. For plasma, urine, or feces, the volume (in milliliters) was used instead of the weight, i.e. values of percent per milliliter are reported instead of percent per gram.

Results

PK. The PK clearance of 125I-CRP was biexponential (fig. 1), with a rapid early phase and a slower later phase. The half-life of 125I-CRP was calculated as 4.7 hr. At the first time point measured, i.e. 5 min, ~50% of injected 125I-CRP was recovered in plasma. In contrast, the clearance of both 125I-mCRP-sol and 125I-mCRP-susp was very rapid; at the 5-min time point, <5% of the injected label could be recovered in plasma (fig. 1, inset).

Plasma samples were treated with TCA to precipitate protein-bound label and separate it from label that might have leached off the protein or remained attached to fragmented protein. At the 4-hr time point, 75% of the label was recovered in the plasma compartment as TCA-precipitable CRP; similarly, 49% and 67% of label was found to be precipitable CRP; similarly, 49% and 67% of label was found to be 75% of the label was recovered in the plasma compartment as TCA-precipitable CRP; similarly, 49% and 67% of label was found to be precipitable CRP; similarly, 49% and 67% of label was found to be precipitable CRP. At the 4-hr time point, 75% of the label was recovered in the plasma compartment as TCA-precipitable CRP; similarly, 49% and 67% of label was found to be precipitable CRP; similarly, 49% and 67% of label was found to be precipitable CRP.

Because plasma was not collected before 5 min, the PK parameters for mCRP could only be estimated based on the residual <5% of the label that remained in plasma after this initial time point. The estimated values for the PK parameters are presented in table 1 to show that, even with the limitations in data analysis, the values for mCRP-sol and mCRP-susp are in close agreement with each other and are markedly different from the values for the more widely studied and more fully understood CRP molecule. Because our calculated values for the CRP conformer agree closely with those from previously reports (Baltz et al., 1985), we consider our model system and methods to be valid and our estimated values for the mCRP conformer to be meaningful.
Because the majority of 125I-mCRP is cleared from the circulation before the first data collection point, the estimated half-life of elimination for 125I-mCRP shown in Table 1 describes only the subfraction of 125I that remains in circulation during the period of 5 min to 24 hr (fig. 1). The AUC and AUMC were calculated to be 10- and 5-fold less, respectively, for mCRP, compared with CRP. The Vss and CLT were 10- and 5-fold greater, respectively, for mCRP, compared with CRP.

**TABLE 1**

PK parameters for 125I-CRP, 125I-mCRP-sol, and 125I-mCRP-susp, estimated by noncompartmental analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CRP</th>
<th>mCRP-sol</th>
<th>mCRP-susp</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (cpm/hr-ml)</td>
<td>2.14 × 10^6</td>
<td>0.25 × 10^6</td>
<td>0.18 × 10^6</td>
</tr>
<tr>
<td>AUMC (cpm-hr-hr/ml)</td>
<td>10.57 × 10^6</td>
<td>2.36 × 10^6</td>
<td>2.01 × 10^6</td>
</tr>
<tr>
<td>Kₑ( hr⁻¹)</td>
<td>0.147</td>
<td>0.050</td>
<td>0.055</td>
</tr>
<tr>
<td>Vₜ (ml)</td>
<td>2.35</td>
<td>23.39</td>
<td>27.64</td>
</tr>
<tr>
<td>CLₜ (ml/hr)</td>
<td>0.474</td>
<td>2.46</td>
<td>2.40</td>
</tr>
<tr>
<td>MRT (hr)</td>
<td>4.95</td>
<td>9.52</td>
<td>11.51</td>
</tr>
<tr>
<td>Mean dose (cpm)</td>
<td>1.01 × 10^6</td>
<td>0.61 × 10^6</td>
<td>0.421 × 10^6</td>
</tr>
</tbody>
</table>

PK parameters were calculated based on plasma 125I activity for all time points (5 and 30 min and 2, 4, 8, and 24 hr).

Values describe only the subfraction of 125I-mCRP remaining in circulation for the 24-hr experimental period.

Because the majority of 125I-mCRP is cleared from the circulation before the first data collection point, the estimated half-life of elimination for 125I-mCRP shown in Table 1 describes only the subfraction of 125I that remains in circulation during the period of 5 min to 24 hr (fig. 1). The AUC and AUMC were calculated to be 10- and 5-fold less, respectively, for mCRP, compared with CRP. The Vₜ and CLₜ were 10- and 5-fold greater, respectively, for mCRP, compared with CRP.

**BD.** Figs. 2 and 3 show the tissue distribution of 125I-CRP and 125I-mCRP-sol, at 4 and 24 hr after iv injection into mice, expressed either as the percentage of the injected dose or as the percentage of the injected dose per gram for each tissue (or organ). At 4 hr, the greatest amount of recovered 125I-CRP was found in plasma (12% of injected dose). Skeletal muscle (7%), liver (6%), skin (5%), and femur bone (5%) were the other predominant sites for the uptake of 125I-CRP (fig. 2A). The greatest concentrations of label (expressed as percentages of the injected dose per gram) were found in the urine and plasma (39%/ml and 14%/g, respectively) (fig. 3A). At 24 hr, the percentage of 125I-CRP in urine was significantly increased (fig. 2A). The rank order of accumulation was urine (22%) > bone marrow (8%) > femur bone (6%) > skeletal muscle (5%). There was a notable increase in the concentration of radiolabel localized to the bone marrow at 24 hr (27%/g), relative to the 4-hr time point (4.9%/g) (fig. 3A).

At the 4-hr time point, the greatest amounts of recovered 125I-mCRP were found in skeletal muscle (10%), skin (8%), bone marrow (6%), liver (6%), femur bone (5%), and plasma (3%) (fig. 2B). No experimental value for urine is reported for this time point because no useable sample was collected. The greatest concentrations of 125I-mCRP-sol were recovered in the bone marrow (21%/g), esophagus (12%/g), spleen (9%/g), and stomach (8%/g) (fig. 3B). At the 24-hr time point, both the percentage and concentration of the radiolabel (fig. 3) indicated substantial localization to the bone marrow (25% and 92%/g, respectively). In addition, a high percentage and a high concentration of radiolabel were recovered in urine (38% and 78%/ml, respectively).

The BD pattern for injected 125I-mCRP-susp was very similar to that described for 125I-mCRP-sol (data not shown). At 4 hr, a higher percentage of injected 125I-mCRP-susp (16%) was recovered in the bone marrow than was measured for 125I-mCRP-sol (6%). There was also greater selective distribution of radiolabel to the bone marrow (56%/g) and urine (36%/ml) at this time point. At 24 hr, further substantial distribution of 125I-mCRP-susp to the bone marrow (92%/g) and urine (55%/ml) was observed. Small amounts of radiolabel were concentrated in the adrenal glands, the gallbladder, and the esophagus.
collected at the 24-hr time point. These data suggest that mCRP, in contrast to CRP, is immediately taken up into tissues within the first few passages through the circulation.

mCRP is a protein that is soluble in low-ionic strength solutions and forms self-aggregates when placed in buffers of physiological ionic strength (Potempa et al., 1983, 1987). Antigens cross-reactive with mCRP are found to be abundantly expressed at the intima, media, and adventitia of a number of normal blood vessels. Immunohistochemical reactivity is also found in the fibrous capsule of the adrenal glands and tonsils, in the fibrous trabeculae of the spleen, and in skin and skeletal muscle (Radosевич et al., 1996). These data are consistent with the view that mCRP is a natural component of the extracellular matrix of reticuloendothelial tissues. The fact that the majority of injected $^{125}$I-mCRP is rapidly removed from the circulation may reflect a strong tendency for the mCRP conformer to partition into tissues where naturally occurring mCRP is found. This result is supported by the large $V_{ss}$ values calculated for mCRP (23–27 ml) and by the BD results. Drugs that are sequestered by tissues exhibit large $V_{ss}$ values (Benet and Sheiner, 1988). CRP, in contrast, is primarily a protein of the circulatory system, having a comparatively small $V_{ss}$ (2.35 ml).

mCRP exhibits an ∼10-fold larger $V_{ss}$ than does CRP, and estimated CL and MRT values were calculated to be ∼5- and ∼2–3-fold greater, respectively, than those for CRP. These data, together with the finding that all radiolabel associated with mCRP could be accounted for at the 24-hr time point, indicate that, by accessing tissues, mCRP is cleared at a slower rate than CRP.

TCA precipitation of plasma samples showed that there was a generally uniform recovery of ∼72–84% of $^{125}$I-CRP from plasma through the 8-hr time point after treatment. However, TCA precipitation of $^{125}$I-mCRP-sol or $^{125}$I-mCRP-susp plasma samples obtained up to the 4-hr time point showed only 49 and 63%, respectively, of the label remaining protein bound (data not shown). The lower percentage of TCA-recoverable label in the <5% subfraction of injected $^{125}$I-mCRP recovered in the plasma suggests that the label might have dissociated from the protein, that mCRP in plasma might have been degraded faster than mCRP partitioned into tissues, or that nonlinear protein binding might have occurred in vivo. These data suggest that the $^{125}$I-mCRP recovered in plasma at 4 hr might be a distinctive subpopulation of $^{125}$I-mCRP or a metabolic breakdown product of the prepared reagent.

Tissue distribution of $^{125}$I-CRP, $^{125}$I-mCRP-sol, and $^{125}$I-mCRP-susp was generally unremarkable, except for the interesting selective BD of both forms of mCRP to the bone marrow. The early distribution measured at 4 hr after iv injection of all forms of $^{125}$I-(m)CRP tested was found in the skeletal muscle, liver, and skin. At 24 hr, whereas 7% (equivalent to 27%/g) of $^{125}$I-CRP was distributed to the bone marrow, ∼25% (equivalent to 92%/g) of both $^{125}$I-mCRP-sol and $^{125}$I-mCRP-susp was accumulated in the bone marrow. Thus, the BD results corroborate the PK estimates that mCRP, to a greater extent than CRP, is rapidly cleared from the blood compartment and enters tissue, especially bone marrow, where a large portion of it remains for at least 24 hr.

It is noteworthy that a key component of the bone marrow is the stroma, which is composed, in part, of reticular proteinaceous fibers that are similar to those found in fibrous tissues of the systemic reticuloendothelial system (Allen et al., 1990; Mohammad and Asai, 1993). The physical characteristics of mCRP as a protein that can self-associate into a matrix-like protein (Motie et al., 1996) may relate to its physiological role as a component of the extracellular matrix of tissues.

The finding that mCRP accumulates in the bone marrow for up to 24 hr after injection is noteworthy in light of our report that mCRP can...
DISTRIBUTION/CLEARANCE OF 125I-CRP AND 125I-mCRP

stimulate megakaryocytopoiesis in mice (Potempa et al., 1996). Drugs that accumulate in a given tissue are believed to serve as a reservoir for prolonged drug action in that tissue or at another site (reached through the circulation) (Benet and Sheiner, 1988). The accumulation of radiolabeled mCRP in the bone marrow agrees with this organ being the site of increased hematopoietic activities and this drug having stimulatory effects on megakaryocytopoiesis. Furthermore, because mCRP can be formed from CRP under a variety of conditions (Potempa et al., 1987), the accumulation of 125I-CRP in vivo in the bone marrow at 24 hr might indicate that some injected CRP was converted in situ to mCRP, which was then localized to this organ. The fact that there was increased accumulation of label in the bone marrow at 24 hr, although there was a very low level of radioactivity associated with mCRP in the circulation, may reflect capacity-limited clearance of mCRP. Because of its low aqueous solubility, injected mCRP may be sequestered (as a noncovalently associated aggregate) into tissues, where it serves as a reservoir for circulating protein. The tissue-associated mCRP would be in equilibrium with the plasma mCRP, which would slowly and continuously be sequestered into specific (e.g. bone marrow) tissues.

Hutchinson et al. (1994) reported the accumulation of 125I-tyramine cellobiose-labeled CRP in hepatocytes of mice and rabbits 24 hr after iv injection. They suggested that the liver is the main organ for pentraxin catabolism. The same group reported that the kidney is the primary organ for excretion of CRP-associated radiolabel in humans (Vigusin et al., 1993). Our results indicate that the final excretory pathway for all forms of radiolabeled CRP appears to be through the urinary tract, presumably after filtration through the kidney. At the 24-hr time point, 40, 78, and 55%/ml of 125I-CRP, 125I-mCRP-sol, and 125I-mCRP-susp, respectively, were found in the urine. We propose that the lack of substantial accumulation of radiolabel in the kidneys may be a sign of the low toxicity associated with the injection of mCRP in both soluble and suspended forms.

This study demonstrates that CRP and both forms of mCRP can be safely injected iv into mice, where they are cleared from the body and excreted in the urine. The selective localization of mCRP to tissues, especially the bone marrow, provides a clue to the bioactivity of this protein as a thrombopoietic factor (Potempa et al., 1996), as well as an amplifying factor in the leukocyte and platelet responses (Potempa et al., 1988). The therapeutic applications of mCRP in the treatment of various immunological diseases are under study.

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References


