Transient Neutropenia: Neutrophil Distribution and Replacement

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Radiolabeled polymorphonuclear (PMN) receptor-specific proteins or peptides lead the list of agents being evaluated for imaging inflammatory foci. Some of these agents induce transient neutropenia. This study was designed to quantify the degree of dose dependency of neutropenia, determine the duration of neutropenia, identify the organs in which these PMNs sequester and ascertain if these PMNs return to the circulation. **Methods:** Rodent anti-PMN (Gr-1) MAb RB6-8C5 (IgG-2a) and Balb/c mice served as the model, and PMN nonspecific ME 31.3 (IgG-2a) as a control. Circulating PMN number was determined several times, 30 min prior to and between 1 min and 120 hr after MAb administration. Iodine-125-MAbs provided quantification of circulating activity and tissue distribution as a function of time. **Results:** Data showed the severity of neutropenia increased with the amount of MAb administered (>95% PMNs lost after 150 μg versus <85% after 10 μg). Moreover, the recovery time for PMN counts to reach the pretreatment level also increased in a dose-dependent manner (96 hr at 150 μg versus 4 hr at 10 μg and 2 hr at 2 μg). The blood activity, however, which declined quickly with the neutropenia, never rose again with PMN recovery. As a function of time, radioactivity in the study group decreased from all organs except from the liver and spleen, whereas in the control group, it decreased from all organs, including the liver and spleen (e.g., 4 hr liver, 29.4% decrease versus 91.2% decrease in the control group; and spleen, 15.5% decrease versus 63.6% decrease in control group). **Conclusion:** The degree and duration of neutropenia is dose-dependent. PMNs, lost from the circulation, sequester in the reticuloendothelial system, and do not return to circulation. Therefore, they are not available to image inflammatory foci. The PMN concentration is restored to a pretreatment level in a dose-dependent fashion, presumably by freshly released PMNs from bone marrow.

Key Words: polymorphonuclear receptor-specific proteins; neutropenia; dose-dependency

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After Metchnikoff's historical definition of inflammation as "a salutary response to some injurious influence," neutrophils have been implicated not only in the pathogenesis of many inflammatory diseases (*1–3*) but have also become the archetypal cells in acute inflammation or infection. During the past two decades, the use of radiolabeled neutrophils has therefore become increasingly popular in the scintigraphic detection of infection and inflammatory processes (*4*). The current technique facilitating this practice, however, mandates an in vitro procedure in which PMNs are separated from the patient's blood and then radiolabeled, either with ^{111}In-oxine or ^{99m}Tc-HMPAO (*4,5*). This procedure is not only time-consuming and requires skill, but also imposes risk to the personnel who handle the patient's blood and to the rare patient who may accidentally receive another patient's blood components.

Recent developments in hybridoma technology and molecular biology have made it possible to identify and prepare proteins and peptides that may interact specifically with PMN surface receptors. Such agents have been labeled, either with or ^{111}In or ^{99m}Tc (*6–17*) and injected directly into the bloodstream of experimental animals or a limited number of patients to selectively label PMNs or other cellular components with the tracer. The hypothesis is that the labeled PMNs in the circulation will then chemotactically migrate to the infectious or inflammatory foci and facilitate localization of the lesions by gamma camera scintigraphy.

Certain neutrophil receptor specific agents are known to induce transient neutropenia in laboratory animals or higher primates (*8,18–20*). In an experiment using monkeys as an animal model and DTPA conjugated with ForNule/FnleYk as a potent chemoattractant to human neutrophils, Fischman et al. (*20*) elegantly demonstrated that the severity of neutropenia was dose-dependent. At the two upper levels of peptide doses (1.0 μg/kg and 10 μg/kg), neutrophils were not allowed to return to the preinjection levels before dosing the animals again. This did not permit them to determine the duration of neutropenia. Furthermore, a peptide of similar mo/wt and charge with no PMN specifically was not used as a control. Additionally, and more importantly, the organs in which the PMNs once lost from circulation sequester as well as whether the PMNs once sequestered return back to the circulation was not determined. This step is vital for their ability to migrate to the inflammatory lesions and permit scintigraphic imaging.

The objectives of this investigation were: to quantify the degree of dose-dependent neutropenia, to determine the time frame over which the neutropenia occurs, to identify the organs in which the receptor agent conjugated neutrophils sequester and to determine if the PMNs once lost from the circulation return to the circulation.

The monoclonal antibody (MAb) RB6-8C5 was used to induce transient neutropenia in mice. RB6-8C5 is a rat IgG2b which recognizes a surface marker (Gr-1) on mature murine neutrophils and eosinophils (*21–27*). RB6-8C5 receptors are also expressed on myeloid cells in the murine bone marrow, but the MAb receptor expression increases as the cells' differentiation into mature granulocyte increases (*27*). In vivo treatment of mice with this antibody selectively depletes neutrophils and eosinophils from the peripheral circulation, but not from organs such as the liver, spleen and lungs (*26*). The circulating number of the other cells, such as the monocyte, macrophages, lymphocytes, erythrocytes and platelets, remain at normal levels.

Experiments performed by others (*23*) have shown that after the single intraperitoneal injection of 150–200 μg RB6-8C5 into mice, severe depletion (>90%) of neutrophils occurred at 24 hr and was maintained for up to 5 days (*23*). By the sixth day, the neutrophil count recovered to its pretreatment level. The mechanism of neutrophil depletion by RB6-8C5 was not known. This PMN specificity therefore rendered RB6-8C5 an ideal prototype antibody, and mice are an affordable animal model for which an adequate number could be killed to examine neutropenia and to perform quantitative tissue distribution

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studies. To further facilitate these studies, RB6-8C5 was labeled with ^{125}I and PMN nonspecific MAb ME 31.3 (IgG-2a) was used as a control.

MATERIALS AND METHODS

Monoclonal Antibodies

Rodent PMN specific MAb RB6-8C5 was separated and purified from mouse ascites induced by RB6-8C5 hybridoma originated in Dr. R. Coffman's laboratory at DNAX Research Institute (Palo Alto, CA). The antibody specificity was examined in Dr. Wayne's laboratory as described by Tepper et al. (*22*). The antibody (2 mg/ml in phosphate buffer saline, pH 7) was divided in 50-μg aliquots and stored frozen until use.

MAb Labeling with Iodine-125

Sodium iodine (^{125}I) solution in 0.01 *M* NaOH was obtained from New England Nuclear DuPont (Billerica, MA). One hundred micrograms of MAb were labeled with ^{125}I by a previously reported iodogen method (*16*). Briefly, 10 μl iodogen solution (1 mg/ml) in $CHCl_3$ were dispensed in a clean conical glass vial and $CHCl_3$ was evaporated with a gentle stream of nitrogen. The MAb was then added, followed by a required quantity of ^{125}I. The vial was closed, the reaction mixture vortexed and the reaction was allowed to proceed for 30 min. The reaction was terminated by the addition of 10 μg sodium metabisulfite and then unreacted ^{125}I was eliminated through Centricon-30 molecular filtration. This step was followed by ITLC and HPLC analyses (*28*). Protein concentration was determined by spectrophotometry ($E^{1\%}_{280} = 14.6$).

Animals and PMN Counting

Albino Balb/c mice (M/F) weighing between 18–23 g were obtained from Taconic LAAS (Germantown, NY) and housed in the institutional Animal Care facility. At least three fresh animals were used per group. Animals were injected with a known amount of unlabeled MAb in 200 μl PBS through a lateral tail vein. At predetermined times, which varied from 60 sec to 96 hr postinjection, unanesthetized animals were placed in a ventilated plexiglass box in such a way that the tail of the animal was easily accessible while the rest of the body remained in the box. A tail vein was then gently punctured with a 27-gauge sterile needle and a drop of blood was allowed to form, which was smeared and stained. At least 300 characteristic white cells (neutrophils, lymphocytes, eosinophils and basophils) were counted under a light microscope and the percentage of neutrophils in the total white cells counted was determined. The base PMN concentration was determined similarly at different times, 30 min before antibody injection.

Dose Response Curve and Controls

Control animals received either 200 μl sterile isotonic saline or an equivalent quantity of the nonspecific antibody ME 31.3, also in 200 μl PBS. To determine the influence of the quantity of RB6-8C5 on the severity and duration of neutropenia, each group of animals received 2, 10, 20, 50 or 150 μg of MAb. Results for each quantity of MAb were then plotted as a percent change in PMN concentration as a function of time.

Tissue Distribution and Blood Clearance of Iodine-125-Labeled MAb

Ten micrograms and approximately 15 μCi ^{125}I RB6-8C5 were injected in a group of three mice. PMN concentration was determined 30 min before and up to 72 hr postinjection as described previously. At each time point, an additional 5 μl blood were carefully pipetted in duplicate from the tail vein blood drop using a calibrated micro pipette. The pipette was washed three times with 0.9% NaCl and the combined samples at each time point were used to determine radioactivity clearance. As a control, 10 μg and approximately 15 μCi ^{125}I ME 31.3 were also administered to a separate group of mice and PMN counts and radioactivity clearance were determined as in the study group.

Separate groups of mice receiving radiolabeled MAbs were killed at 1, 15, 30, 120 and 240 min postinjection and their tissues were dissected and weighed. Radioactivity distribution as the percentage administered dose per gram of tissue was determined.

Histology

Livers from animals receiving 150 μg RB6-8C5, 150 μg ME 31.3 and 0.9% NaCl were dissected 2 hr postinjection, preserved in 10% formalin and then thin sections were embedded in parafin. Histologic sections were then prepared and stained with hemotoxylin and eosin.

RESULTS

Neutropenia occurred consistently with all administered quantities of RB6-8C5. Depending upon the quantities of the MAb administered, there were distinct differences. As the quantity of MAb increased, the severity of neutropenia increased and the time it took to restore the PMN count to pretreatment levels was also prolonged. For example, with 150 μg RB6-8C5, >95% of the PMNs were depleted from the circulation. Forty-eight hours later, the PMN counts were only 65% and it took nearly 4 days to restore the PMN count to the pretreatment level. On Day 5, the PMN count reached 130% of the pretreatment level. At 20 μg, a nadir of 7.6% was reached in 1 hr, which bounced back to the normal value in 2 days. On Day 3, the PMN level went up to 238% of the preinjected level. With 10 μg RB6-8C5, a nadir of 11.7% was reached in 30 min and the PMN count returned to the pretreatment level within 4 hr. With 2 μg RB6-8C5, the nadir was 23.7% and the pretreatment PMN concentration levels were attained in 2 hr (Fig. 1). With the injection of control agents (e.g., saline or nonspecific MAb ME 31.3), the loss of PMN from the circulation was minimal (<10%) and normal levels were reached within 30 min postinjection. In these animal study groups, the PMN level continued to increase and reached approximately 200% by the 4 hr (Fig. 2). By Day 4, the levels returned to the preinjection concentration. These results are plotted as a function of time in Figures 1 and 2.

For blood clearance and tissue distribution studies, 10 μg ^{125}I RB6-8C5 were administered intravenously. Blood clearance of radioactivity shown in Figure 2 demonstrates that the initial loss of radioactivity from the circulation was consistent with the loss of PMN from the circulation. Unlike the PMN count, however, which returned to the pretreatment level by 4 hr postinjection, the radioactivity in the circulation did not. These levels of circulating radioactivity, most of which was bound to plasma protein, were consistently lower than those of the ^{125}I-labeled control MAb ME 31.3. These data indicate that PMNs that had interacted with radiolabeled RB6-8C5 were removed from the circulation and were probably sequestered in other organs.

Tissue distribution data obtained from 1 min to 4 hr postinjection (Figs. 3 and 4) showed similar clearance of radioactivity from whole blood as well as from the lungs and kidneys, but not from the liver and spleen. Data shown in Figure 4A, which presents the distribution of ^{125}I-RB6-8C5 and ^{125}I-ME 31.3 (control) in the liver and spleen of separate groups of Balb/c mice, depicts a rapid clearance of radioactivity from both the liver and spleen in the control group but not from the liver and spleen of the animals receiving the PMN-specific ^{125}I-RB6-8C5. At 4 hr postinjection, for example, the liver uptake in the animals receiving ^{125}I-RB6-8C5 decreased only to 70.6% ± 1.2% from the uptake at 1 min postinjection as compared to that of 9.8% ± 1.1% in the ME 31.3 control group ($p < 0.01$).

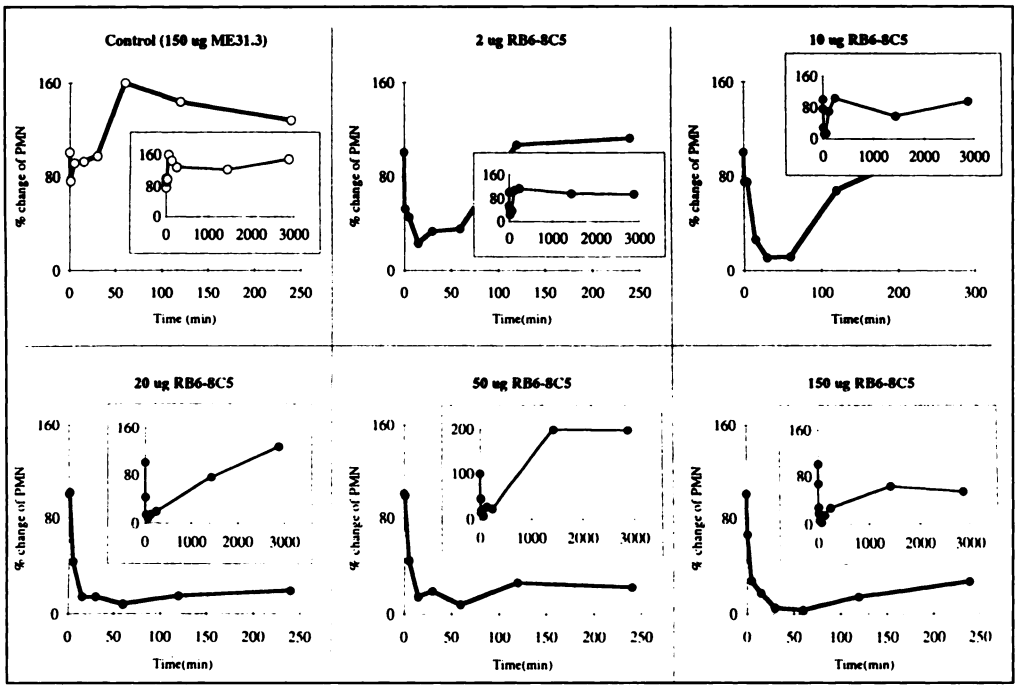

FIGURE 1. Composite of percent change in circulating PMN as a function of RB6-8C5 quantity given intravenously. For each quantity of MAb, an expanded short (0–250 min) and long time (inset, 0–300 min) response are presented. As can be seen, the circulating PMN number began to decrease almost instantaneously with all MAb quantities (except the control, MAb ME 31.3, 150 μg) and came to a nadir within approximately 10 min. Also, as the administered MAb quantity increased, the time for the PMN to reach preinjection concentration increased.

Similarly, splenic uptake in the study group also decreased only to 84.5% ± 1.0% as compared to that of 36.4% ± 1.3% in the control group. In contrast, as shown in Figure 4B, radioactivity cleared, as a function of time, from all other prominent organs of animals receiving either RB6-8C5 or ME 31.3.

A composite of photomicrographs of histological slices (Fig. 5) show infiltration of inflammatory cells in the liver of animals receiving RB6-8C5 but not in the liver of animals receiving either ME 31.3 or 0.9% NaCl. These findings were consistent with quantities of radioactivity found in the livers of animals receiving the two ^{125}I MAbs. These data suggest that the PMNs interacting with ^{125}I-RB6-8C5 sequester in the liver and spleen and do not return to the circulation.

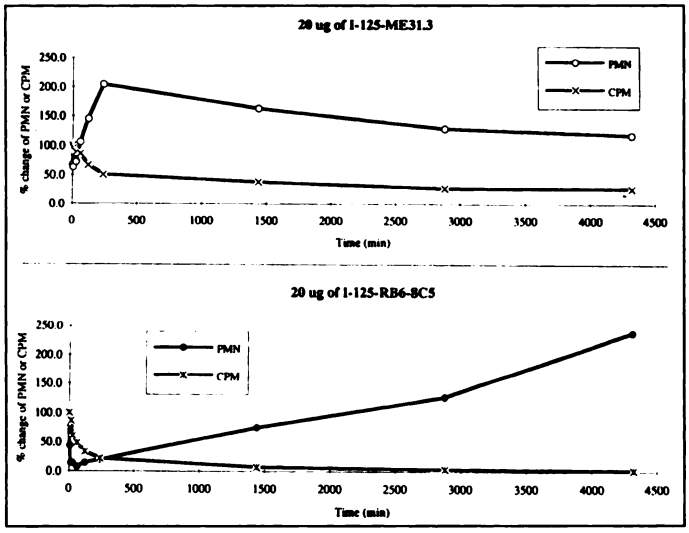

FIGURE 2. Composite of percent change in PMN and radioactivity (CPM) in circulating blood. With ^{125}I-labeled PMN-specific RB6-8C5 MAb (lower panel), the radioactivity cleared rapidly and so did the PMN. As a function of time, the PMN number increased but not the radioactivity, thereby indicating the loss of labeled PMN in the liver and spleen (Fig. 3). In contrast, with ^{125}I-labeled control MAb (^{125}I-ME 31.3, upper panel) the radioactivity in the circulation decreased but the PMN did not.

DISCUSSION

The delivery of PMNs to the site of inflammation involves a series of complex biochemical processes mediated by chemotactic stimuli and include adherence to the vascular endothelium, diapedesis through vascular walls and directional migration toward the chemotactic gradient.

A gamma-emitting radioactive compound that will enable investigators to label PMNs selectively in vivo will be a highly useful agent in the biological sciences because such an agent will:

1. Permit investigators to perform pharmacokinetic studies in animals and humans for better understanding of pathophysiology of disease.
2. Eliminate the drawbacks of current ex vivo labeling procedures.
3. Simplify the technique of scintigraphic imaging of inflammatory foci.

For this purpose, many years ago, our attention was drawn to a potent synthetic chemotactic peptide, formyl-Met-Leu-Phe or FMLP (*6,18*). After labeling with ^{111}In, the specificity for the

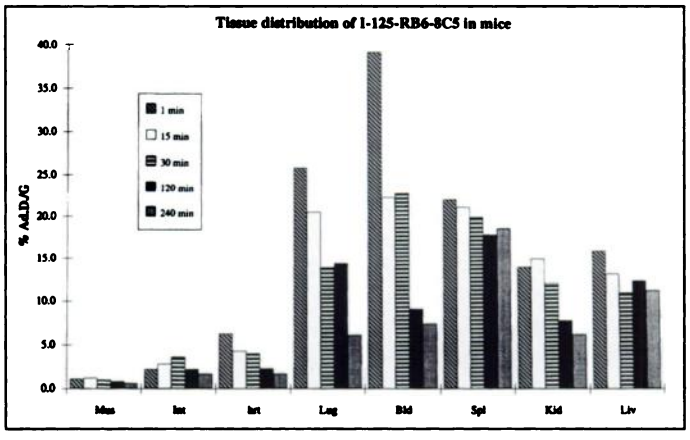

FIGURE 3. Tissue distribution of ^{125}I-RB6-8C5 MAb in mice. The histograms show that as the time after injection elapsed, the radioactivity in all organs, except in the liver and spleen, decreased (Fig. 4A).

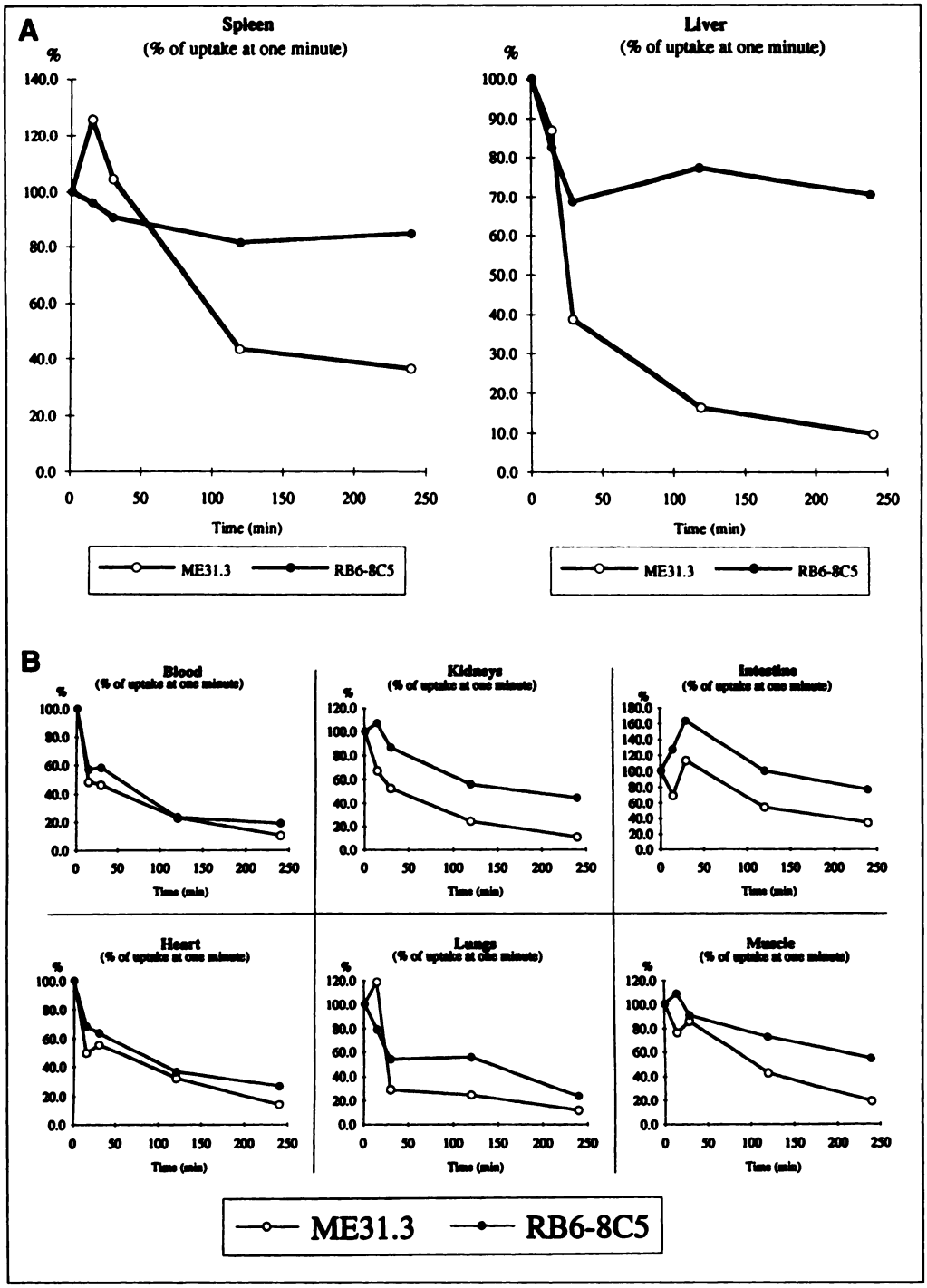

FIGURE 4. (A) Right graph shows ^{125}I-RB6-8C5 and ^{125}I-ME 31.3 clearance from the liver; left graph shows splenic clearance. Both graphs depict little or no clearance of ^{125}I-RB6-8C5 radioactivity from these organs compared to ME 31.3. (B) Radioactivity clearance as a function of time from some prominent organs of animals (except the liver and spleen, shown in (A) receiving ^{125}I-RB6-8C5 or ^{125}I-ME 31.3 (control). There is radioactivity clearance from these tissues, regardless of the antibody received.

human PMN was excellent. Data indicating that as little as 0.5 μg of FMLP-induced transient neutropenia in rabbits (*19*) discouraged us from further investigation. With renewed interest in ^{111}In and ^{99m}Tc-FMLP, corroborating data were presented which confirmed neutropenia in rabbits and monkeys (*20*). The disposition of PMNs lost from the circulation and, more importantly, their reappearance in circulation, if any, remained unknown. Reappearance into the circulation of PMNs labeled with a radioactive agent would be essential for them to migrate into inflammatory lesions and to enable investigators to image the lesion by that mechanism.

Although the MAb RP-3 is known to induce neutropenia in the rat (*29*), we chose a well-characterized MAb, RB6-8C5, that was available to us and is known to cross-react with rodent PMN-surface glycoprotein GA-1 (*20–26*). The choice of this MAb made it easier for us to label the protein with ^{125}I than labeling FMLP with ^{111}In or ^{99m}Tc and provided us with a mouse model that was practical and affordable for performing the studies described.

These studies allowed us to make some important and original observations pertinent to the use of PMN-specific compounds intended as agents for imaging inflammatory processes, and perhaps for examining the pathogenesis of certain diseases. We observed that the severity of neutropenia was dose-dependent, and that as the concentration of the agent increased the state of neutropenia was prolonged, taking up to 4 days to restore the preinjection PMN concentration at 10^{-9} *M* (150 μg) dose of the MAb. At less than a 10^{-10} *M* (10 μg) MAb dose, a nadir of 11.7% was reached. The PMN count, however, returned to pretreatment levels within 4 hr.

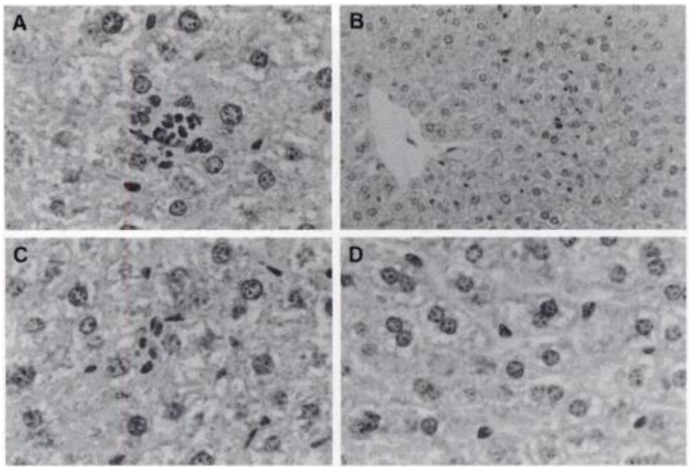

FIGURE 5. Photomicrographs of the livers of animals receiving 150 μg RB6-8C5 (A, 1000×; B 400×), 150 μg control MAb ME 31.3 (C, 1000×) and 0.9% NaCl (D, 1000×). Panels A and B (MAb RB6-8C5) show a number of neutrophils, both scattered individually or in small aggregates, but not panels C or D.

The data also strongly suggest that PMNs once lost from the circulation are taken up by the reticuloendothelial system, primarily the liver and spleen, and do not return back to circulation. This phenomenon, if reproducible in humans, would severely hamper the ability of such agents to permit imaging of inflammatory foci by that mechanism.

We believe that the PMNs that restore the cell concentration in peripheral blood must be released from the bone marrow. The higher MAb doses must interact with myelocytes, PMNs alone or with both in the bone marrow to adversely affect the recovery process. Anti-RB6-8C5 antigen expression on myelocytes has been observed previously (*28*). Due to the difficulty in harvesting large enough quantities of bone marrow from these small animals, we could neither count the PMN concentration in the bone marrow before or after the administration of the agent, nor could we ascertain if the labeled PMNs were taken up by the bone marrow.

The underlying mechanism by which these agents induce neutropenia is not clearly understood. Increased PMN adherence, changes in PMN membrane surface charges and changes in chemotactic factors as well as PMN shape and size have been proposed as the likely reasons (*18*). Although the liver and spleen uptake remained practically unchanged in our studies, the uptake in all other organs continued to diminish over the period of our observation (4 hr). Contrary to this observation, the radioactivity in all organs from animals receiving the nonspecific MAb continued to decline as a function of time. We limited this observation period to 4 hr, since we only wanted to observe cell behavior during the short time in which the neutropenia occurred and PMN concentration was restored.

We also observed that that the PMN counts reached approximately 200% of the initial concentration with the administration of saline and a nonspecific antibody, indicating the burst of PMNs from bone marrow. The underlying pathophysiology of this phenomenon is not clearly understood.

An additional concern with the use of such agents in humans is that the sudden loss of PMN by hyperadherence, and possible consequent agglutination, may lead to capillary stasis and potentially to organ dysfunction. One possible approach to preventing neutropenia is to administer extremely high-specific activity preparations that will deliver a suitable quantity of radioactivity to a low enough number of PMNs so that neutropenia may not be induced. Calculations based on the observation of Fischman et al. (*20*) indicated that as little as 0.7 μg FMLP will induce neutropenia in humans. Preparations containing less than 0.7 μg and 25 mCi ^{99m}Tc may be difficult to achieve on a routine basis.

There are at least three known neutrophil-specific MAbs, MCA-480 (IgM), BW 250/183 (IgG2a) and MN-2 ($F(ab')_2$, being investigated to image abscesses in humans (*7,10,12*). Whether or not they induce neutropenia is not known. It may, however, be important to administer only controlled quantities of MAb that may bind avidly to PMNs. Knowing the kd values and the number of receptors per PMN, specific for MCA-480, and assuming that the MAb molecules will uniformly bind to each circulating PMN, we had estimated that 100 μg MAb will saturate only 0.4% of the available receptors per cell and would not stimulate the PMNs (*7*). Although this degree of receptor saturation does not ensure the lack of neutropenia, it provides a basis upon which data may be generated for future analysis.

Furthermore, over the past few years, it has been known that many anti-human platelet antibodies that recognize either the CD9 antigen or GPIIb-IIIa complex, two of the most abundant platelet surface glycoproteins, cause platelet aggregation accompanied by a full activation response (*30*). Upon administration of one such anti-human platelet MAb, P-256, labeled with ^{111}In, an abrupt loss of circulating radioactivity occurred, as reported by Lavender et al. (*31*). This may indicate transient thrombocytopenia. Apparently, it is encouraging to note that thrombocytopenia was not observed in Phase I clinical trials with ^{99m}Tc-P-280, a peptide specific for activated GPIIa-IIIb receptors (Marcus CS, *personal communication*, 1995). Nonetheless, since many radiolabeled proteins and peptides for in vivo binding of neutrophils and platelets are being investigated as agents for imaging infectious foci or vascular thrombi, a systematic study ellucidating this phenomenon by each agent is warranted.

Indeed, subsequent to the initial preparation of this article, Hosono et al. (*32*) reported that thrombocytopenia occurred in animals receiving $NNKY_2$-11 MAb specific for GPIIb-IIIa-complex receptors on platelets. Their observations also corroborated our findings in that the thrombocytopenia was dose-dependent and that the radiolabeled-MAb bound platelets were sequestered by the liver and spleen and were not released into the circulation, even when peripheral platelet counts returned to normal levels.

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