PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **163**, 126–131 (1980)

The Quantitation of Hemorrhage in the Skin. Measurement of Hemorrhage in the Microcirculation in Inflammatory Lesions and Related Phenomena[1] (40733)

MARGARET M. KOPANIAK,[2] ANDREW C. ISSEKUTZ,[3] CLEMENT E. BURROWES, AND HENRY Z. MOVAT[4]

Division of Experimental Pathology, Department of Pathology, Medical Sciences Building, University of Toronto, Toronto, M5S 1A8 Canada

The acute inflammatory reaction has been extensively studied and several of its parameters quantitated. The most thorough measurements involved changes in blood flow, vascular permeability, and leukocyte infiltration. Although hemorrhage has often been observed in inflammatory reactions, to our knowledge, no satisfactory technique has been developed which is sensitive enough to quantitate it in cutaneous lesions. However, hemorrhage into soft tissues can be quantitated as shown by Schertzer and Ward (1) by homogenizing the tissue, and, following extraction, estimating the hemoglobin concentration spectrophotometrically. A similar approach was attempted in our laboratory. Hemoglobin was extracted from hemorrhagic skin lesions by the method of Dodge *et al.* (2), but this method proved to be too insensitive in our system. A second approach was to use ^{51}Cr or ^{111}In to label red blood cells (RBCs) *in vitro* followed by their reinfusion. These techniques proved feasible, but sensitivity of the assay was greatly improved when RBCs were labeled *in vivo* with ^{59}Fe. In initial experiments the RBCs were labeled in the rabbits in which dermal lesions were induced. Subsequently the RBCs of a donor rabbit were labeled *in vivo* and then transfused into recipient rabbits. Hemorrhagic lesions were induced in the skin of recipient rabbits by injections of proteolytic agents such as a lysate of human neutrophil leukocyte lysosomes (3) and trypsin, or microbial agents, such as formalin-killed *Escherichia coli*. Quantitation of hemorrhage in the local Shwartzman reaction was also attempted. Both methods, the direct *in vivo* ^{59}Fe-labeling of RBCs and the transfusion technique are described in this paper.

Materials and methods. In vivo RBC-labeling. Female New Zealand albino rabbits (2.5–3 kg) obtained from the same breeder were used in all the experiments. [^{59}Fe]Ferrous citrate (26 μCi/mg) in 1% citric acid was obtained from New England Nuclear (Lachine, Quebec). Rabbits were made slightly anemic by bleeding (20–30 ml) from the central ear artery. The collected blood was allowed to clot and the serum obtained after two centrifugations (200*g*, 10 min, and 1100*g*, 10 min) was used for incubation with [^{59}Fe]ferrous citrate according to the method described by Hodgson *et al.* (4). Fifty microcuries of ^{59}Fe were incubated in 5 ml autologous serum for 10 min at 23°C. After the incubation the serum was reinjected into the marginal ear vein of the rabbit. Blood samples were taken every 24 hr to determine the amount of radioactivity localized in the RBC fraction vs the plasma fraction. To separate the plasma from the RBC-mass, the blood (containing 10 IU/ml heparin) was centrifuged at 500*g* for 10 min. The plasma was taken off and the RBC were washed with 2 vol of sterile 0.9% saline and centrifuged again (500*g*, 10 min). The radioactivity was counted using an Intertechnique γ-spectrometer (Canatech Inc., Toronto, Ontario). The percentage of cell-bound radioactivity was determined by dividing the counts per minute (cpm) in the RBC

---

[1] Supported by Grant T. 1-15 from the Ontario Heart Foundation.

[2] Graduate student supported by the Medical Research Council of Canada.

[3] Postdoctoral Fellow of the Medical Research Council of Canada.

[4] Career Investigator of the Medical Research Council of Canada.

0037-9727/80/010126-06$01.00/0

Copyright © 1980 by the Society for Experimental Biology and Medicine.
All rights reserved.

fraction by the total counts per minute in the unfractionated blood sample. The rabbits were used for inducing dermal inflammatory sites 3–4 days following the injection of [^{59}Fe]ferrous citrate.

^{59}Fe-Labeled RBC transfusion. Rabbits were made slightly anemic as already described and 300 μCi of [^{59}Fe]ferrous citrate (preincubated in 5 ml of serum) were injected into the marginal ear vein. Three to four days after labeling the radioactivity per milliliter of blood was determined and enough anticoagulated (10 IU/m/heparin) blood was collected to contain approximately 20 $\times$ 10^{6} (wide open spectrometer window) or 6 $\times$ 10^{6} cpm (on 500–2000 keV window setting). The ^{59}Fe-RBCs were lightly packed (200*g*, 10 min) and infused into the recipient rabbits' circulation. The recipient rabbit's serum was previously cross-matched with the donor's RBC for agglutinating antibody (50 μl of 0.5% RBC in veronal-buffered saline were incubated with 50 μl of recipient's serum at 37°C for 45 min) and checked for hemolysis and rouleaux formation under the microscope. The amount of radioactivity per ml of circulating blood was determined and this allowed conversion of counts per minute per lesion into equivalent number of microliters of extravasated blood.

Intradermal lesions. Intradermal lesions were induced on the backs of rabbits after the hair had been removed with electric clippers. The transfused ^{59}Fe-RBC were already in the circulation at the time of induction of the first lesions.

Two-tenths milliliter of heat-killed *E. coli* (5 $\times$ 10^{9}/site) (Strain X, Medical Teaching Laboratories, University of Toronto) was used to induce inflammation in *in vivo* labeled rabbits. A different batch (Strain BAM, Med. Teach. Labs., University of Toronto) of formaldehyde-killed *E. coli* (6 $\times$ 10^{8}/0.2 ml/site) was injected intradermally in the transfused rabbits. *E. coli* were injected at varying times prior to sacrifice so that lesions of different ages were present at death.

A lysate of human polymorphonuclear leukocyte lysosomes (PMN-lysate) (425 *µM*/min/ml BOC-alanine esterase activity) (3) was injected intradermally 1 hr before sacrificing the rabbit (*in vivo* labeled rabbits were used). Injections (0.2 ml) were made of 1:3 and 1:9 dilutions. Trypsin (TPCK, Worthington Biochemicals, Freehold, N.J.) was injected in 200-, 100-, and 50-μg doses and the lesions also allowed to develop for 1 hr before sacrificing the animal.

Local Shwartzman reactions were induced by injecting 100, 50, 25, 12.5, and 6.25 μg of *Serratia marcesens* endotoxin (3130-25B, Difco, Detroit, Mich.) in 0.2 ml followed by an intravenous injection of 100μg 18 hr later. The animals were killed 6 hr after the iv injection.

In order to standardize the hemorrhage assay, microliter amounts of the circulating radioactive blood were diluted in saline and 0.2 ml injected intradermally.

To ascertain that the circulating ^{59}Fe-RBC were indeed measuring hemorrhage and not the magnitude of blood flow, 0.1 and 1 μg of prostaglandin E_2 (PGE_2) were injected per site and allowed to develop for 30 min.

Control sites were either saline injected or uninjected skin. Both contained identical amounts of radioactivity. The rabbits were sacrificed with an intravenous overdose of sodium nembutal. The skin was removed and blood was expressed manually from the larger vessels to reduce variations in the background of the skin. The lesions were punched out with a cork borer (1.5 cm in diameter) and the radioactivity in the discs of skin counted.

The γ-spectrometer window was set on either a wide open channel (^{59}Fe-gross channel) or on 500–2000 keV, as indicated in the figures. In the calculations of the data, γ-spectrometer background counts per minute were subtracted from each sample.

Results. The incorporation of ^{59}Fe into RBC was studied in several rabbits. In a typical pattern, 24 hr after the intravenous injection of 50 μCi of ^{59}Fe, there were 150,000 cpm/ml (on the ^{59}Fe-gross channel) circulating in the blood. At 48 and 72 hr 210,000 cpm/ml were circulating with >99% of the radioactivity localized in the RBC-fraction. Seven days after injection of ^{59}Fe the radioactivity in blood dropped to 191,000 cpm/ml. Since the plateau of ^{59}Fe

incorporation occurred approximately 3 days after ^{59}Fe injection, the experiments were performed at this time. Similarly, in the RBC-transfusion experiments, ^{59}Fe-RBCs were obtained from the donor 3–4 days after ^{59}Fe injection.

The development of hemorrhage after intradermal injections of heat-killed *E. coli* (5×10^9/site) was studied in rabbits whose own RBCs were labeled with ^{59}Fe and in transfused rabbits. Figure 1 shows the combined results from three rabbits whose own RBCs were labeled. The number of microliters of extravasated blood at the sites was calculated by dividing the counts per minute per site by the counts per minute per microliter of circulating blood. The control skin sites were found to contain the radioactivity equivalent to 4.0 ± 0.5 μl (SEM) of blood. The rate of ^{59}Fe-RBC accumulation was greatest between 3 and 4 hr after the injection of *E. coli*. The number of ^{59}Fe-RBCs in the lesions remained unchanged between 4 and 8 hr but a decrease in ^{59}Fe radioactivity localized at the sites was observed at 24 hr. The maximal amount of extravasated blood found in a lesion was 36.2 ± 2.5 μl (SEM).

Formalin-killed *E. coli* (6×10^8/site) were injected intradermally into rabbits transfused with ^{59}Fe-RBC (Fig. 2). The control skin sites contained 1.1 ± 0.1 μl (SEM) of blood. Hemorrhage increased up to 6 hr after the injection of bacteria. No further increase in ^{59}Fe-RBC at the sites was detected after 6 hr. The maximal localization of ^{59}Fe-RBC occurred at 6 hr and corre-

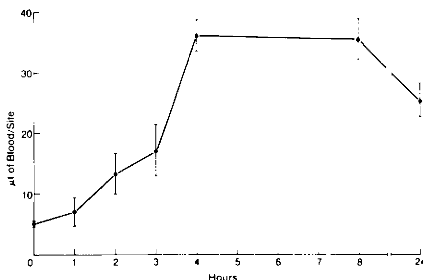

FIG. 1. Lesions induced in the dermis of rabbits by injecting 0.2 ml of *E. coli*, 5×10^9 organism per site. The animals had been injected 3 days before with ^{59}Fe, as described under Materials and Methods. The samples were counted on ^{59}Fe-gross channel.

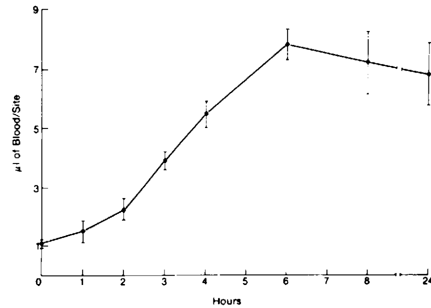

FIG. 2. Similar lesions to those in Fig. 1, except that the rabbits were transfused with ^{59}Fe-labeled red cells and only 6×10^8 *E. coli* per site was injected. The γ-spectrometer window was set at 500–2000 keV.

sponded to 8.0 ± 0.45 μl (SEM) of extravasated blood. The overall kinetics of hemorrhage development were similar in the *in vivo* labeled and in the transfused rabbits.

The results obtained with a PMN-lysate and trypsin are shown in Table I. Visually a very rapid development of hemorrhage was observed in both PMN-lysate and trypsin-induced lesions. With the highest dose of PMN-lysate (85 μM/min/ml BOC-alanine esterase activity) and with the highest dose of trypsin (200 μg) an approximately 10-fold increase in ^{59}Fe-RBC localization over normal skin controls was measured. The magnitude of hemorrhage was directly proportional to the dose of the proteases injected. In these experiments the *in vivo* method was used. The PMN-lysate was tested also with the transfusion method. With the latter the hemorrhagic lesions were similar to those in Table I. However, the control (saline) sites were considerably lower (1.2 ± 0.1 μl).

Figure 3 shows the correlation between the amount of radioactive blood (withdrawn from the animal's own circulation) injected intradermally and the radioactivity measured. The experiment was performed in a ^{59}Fe-RBC transfused rabbit and the radioactivity measured on the 500–2000 keV channel. A linear relationship exists as seen in the figure.

In order to determine if ^{59}Fe-RBC measured hyperemia in the lesions rather than hemorrhage, PGE_2 was injected intradermally. It has previously been shown that the hyperemia in the skin was over 10-fold

TABLE I. QUANTITATION OF HEMORRHAGE IN THE RABBIT'S SKIN INDUCED BY HUMAN PMN-LYSOSOMAL LYSATE OR TRYPSIN[a]

Material injected	Dose	^{59}Fe-cpm[b] ± SEM (n = 5)	Blood (μl-eq)
Saline control	—	1332 ± 193	4.0 ± 0.58
Human PMN-lysate	85[c]	13443 ± 3245	40.4 ± 9.75
	28	5711 ± 472	17.2 ± 1.42
	9	4274 ± 1110	12.8 ± 3.32
Trypsin (μg)	200	15712 ± 3415	47.2 ± 10.26
	100	7297 ± 1351	21.9 ± 4.05
	50	3227 ± 827	9.7 ± 2.48

[a] Various concentrations of PMN-lysate or trypsin were injected intradermally into rabbits 1 hr before sacrifice. The rabbits had circulating ^{59}Fe-RBCs as described under Materials and Methods. The counts in excess of control skin are due to RBC extravasation. The *in vivo* method was used.
[b] As measured on ^{59}Fe-gross channel.
[c] μM/min/ml of BOC-alanine-esterase activity.

greater than normal skin 20–40 min following the injection of PGE_2 (5, 6). If ^{59}Fe-RBCs were measuring blood flow increases, there should have been localization of ^{59}Fe radioactivity in the PGE_2-induced hyperemic lesions. These lesions, however, showed no significant difference from control skin sites. It should be noted that at the end of each experiment the blood was expressed peripherally from the vessels as described under Materials and Methods.

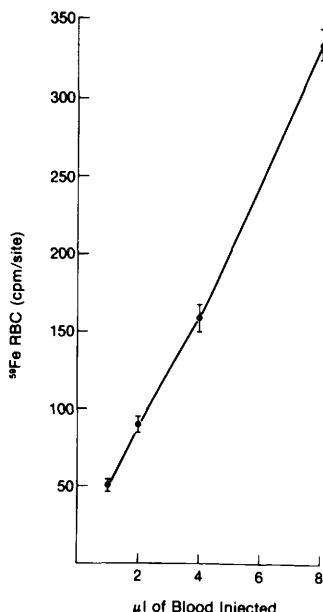

FIG. 3. The relationship between the volume of radioactive blood injected intradermally and the cpm measured. The ^{59}Fe radioactivity was counted on 500–2000 keV window.

Attempts were made to ascertain that of all blood components only RBCs were specifically labeled by ^{59}Fe. After centrifugation of the whole blood from donor rabbits at 500g, the platelet-rich plasma consistently contained <1% of the total counts per minute. After repeated hydroxyethyl cellulose sedimentation of RBC (7) and ammonium chloride lysis of the contaminating RBCs the leukocyte fraction contained counts which were not significantly higher than γ-counter background, although 40 ml of blood containing 6 × 10^6 cpm were fractionated.

Table II shows the results obtained in experiments in which the cutaneous Shwartzman reaction was elicited.

Discussion. The inflammatory reaction induced by intradermal injections of certain proteolytic enzymes, bacterial lipopolysaccharides, gram negative or positive bacteria, and the formation of antigen–antibody complexes, as in the cutaneous Arthus reaction is associated with visible hemorrhage (8). Although the hemorrhages which occur in the microcirculation are clearly visible, the small amount of RBC extravasation is often difficult to quantitate. In this study, quantitation of extravasation of blood was attempted by using radiolabeled red cells. Initially ^{51}Cr was used to label autologous RBCs *in vitro*,

TABLE II. QUANTITATION OF HEMORRHAGE IN THE LOCAL SHWARTZMAN REACTION[a]

Dose of endotoxin injected	^{59}Fe-cpm ± SEM (n = 3)	Blood (μl-eq)
100	2708 ± 163	36.75 ± 2.21
50	891 ± 60	12.56 ± 0.85
25	593 ± 32	8.04 ± 0.43
12.5	485 ± 74	6.03 ± 0.92
6.25	330 ± 19	4.06 ± 0.23
Saline control	234 ± 7	2.55 ± 0.08

[a] The local Shwartzman reaction was elicited on the backs of rabbits as described under Materials and Methods. The samples were counted on 500–2000 keV γ-spectrometer window. The transfusion method was used.

followed by their reinjection into the original rabbits' circulation. In other experiments, ^{111}In was used to label the RBCs. In both cases the assay was not sensitive enough to distinguish small differences between lesions.

Rabbits whose RBCs were labeled *in vivo* were then used and found to yield consistent results. Time course studies of the development of hemorrhage could be performed over a 24-hr period since the ^{59}Fe-labeled RBCs were continuously present in the circulation. During the 24 hr of these experiments, the RBC-bound radioactivity did not change. The *in vivo* labeling technique allows long-term experiments (up to 4–5 days) to be performed because of the nearly constant level of circulating radioactivity in red cells. The number of free or plasma-associated counts remained less than 1% of the total counts in blood. This very low percentage of non-red cell-bound counts is the major advantage of the ^{59}Fe-labeling technique. When ^{51}Cr or ^{111}In-labeling was employed 3–6% of the radioactivity was plasma associated and this probably diminished the sensitivity of these techniques.

However, *in vivo* labeling of RBCs with ^{59}Fe does present certain disadvantages. Since ^{59}Fe is injected intravenously it is readily available to any cell for uptake. The ^{59}Fe may be bound to or incorporated by cells in the skin, including macrophages and dermal and epidermal cells. This may account for the higher counts and therefore blood equivalents in control skin in *in vivo* labeled rabbits (4.0 ± 0.5 μl, Fig. 1) than in rabbits which were transfused with ^{59}Fe RBCs (1.1 ± 0.1 μl; Fig. 2). This decrease in the background radioactivity of normal skin in transfused rabbits was achieved even though the circulating counts per minute per milliliter of blood in RBCs was similar to that in the rabbits labeled *in vivo*. The reduction in background counts allowed a much greater sensitivity in measuring the cutaneous hemorrhages. At the same time the plasma-associated radioactivity was further decreased to approximately 0.2% of the circulating radioactivity because most of the plasma in the transfused blood was discarded prior to transfusion. A disadvantage of the transfusion method in the long-term experiments is a steady decline in circulating ^{59}Fe-RBCs with time. In 48-hr-long experiments there was a 15% decrease in ^{59}Fe radioactivity between the time of transfusion and the end of the experiment. However, counts in plasma were not increased. It may be concluded that for long-term experiments the *in vivo* labeled rabbits are more suitable, as their ^{59}Fe-RBCs remain at stable levels in the circulation. If however, sensitivity of the assay is of primary importance, the transfusion method seems more advantageous. Therefore in studies dealing with the quantitation and kinetics of the acute inflammatory reaction induced in the skin of rabbits with killed *E. coli*, in which four parameters of the reaction (blood flow, vascular permeability, leukocyte infiltration, and hemorrhage) were measured, the transfusion method was used to quantitate hemorrhage (9).

The hemorrhage assay developed proved very useful in quantitation of cutaneous

hemorrhage. It should also be possible to measure hemorrhage in other organs using one of the methods described. Preliminary studies in our laboratory indicate that hemorrhage in the lung can be successfully quantitated using ^{59}Fe-labeled RBCs.

Whereas the blood can be removed from the vessels of the lung by perfusion of the organ. In the skin this is not feasible and therefore it was found essential to express the blood manually by "massaging" the skin along the visible vessels toward the periphery until they looked empty. Without this procedure the control sites showed variable radioactivity.

Summary. Cutaneous hemorrhage was quantitated using ^{59}Fe-labeled red blood cells. The red blood cells were labeled by injecting ^{59}Fe-ferrous citrate, preincubated with serum. Skin lesions were induced either in rabbits whose own red cells had been labeled or in animals which had been transfused with labeled cells. Hemorrhagic lesions were produced by injecting heat- or formalin-killed *E. coli,* lysates of human PMN-leukocyte lysosomes, trypsin or by eliciting the local Schwartzman reaction.

1. Schertzer, H., and Ward, P. A., Amer. Rev. Resp. Dis. **117**, 551 (1978).
2. Dodge, J. T., Mitchell, C., and Hanahar, D. J., Arch. Biochem. Biophys. **100**, 119 (1963).
3. Janoff, A., Biochem. J. **114**, 157 (1969).
4. Hodgson, G., Eskuche, I., Yudilevich, D., Hernandez, P., and Toha, J., Proc. Soc. Exp. Biol. Med. **96**, 826 (1957).
5. Johnston, M. G., Hay, J. B., and Movat, H. Z., Ag. Actions **6**, 705 (1976).
6. Kopaniak, M. M., Hay, J. B., and Movat, H. Z., Microvasc. Res. **15**, 77 (1978).
7. Kerry, P. J., Res. Veter. Sci. **21**, 356 (1976).
8. Movat, H. Z., "Inflammation, Immunity and Hypersensitivity. Cellular and Molecular Mechanisms," 2nd ed., p. 1, Harper & Row, Hagerstown, MA (1979).
9. Kopaniak, M. M., Issekutz, A. C., and Movat, H. Z., Amer. J. Pathol., in press.

Received July 31, 1979. P.S.E.B.M. 1980, Vol. 163.