^{51}Cr LABELING OF CONCENTRATED PHAGOCYTES

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Occult infections remain a difficult diagnostic problem in clinical medicine. Isotope tagging of white cells has been used. This paper describes a rapid and simple technique for isolating large numbers of purified phagocytes that are by all parameters intact. Parameters involved in obtaining an efficient and consistent tag with ^{51}Cr are described. This technique has potential value in the study of leukocyte kinetics.

Occult infection remains a perplexing and difficult clinical problem. Often the physician is clinically suspicious of infection, yet conventional methods—physical examinations, white blood cell count, and x-rays—are either nondiagnostic or do not delineate a suspected infection. Thus the physician can alternatively recommend blind surgical exploration or he can delay, for better or worse, until the infection progresses and becomes clinically unequivocal. Improved, noninvasive techniques for locating infections would have immeasurable importance in clinical medicine.

In 1968 Winkelman, et al (*1*) reported visualizing a purulent abscess in a rabbit using ^{51}Cr-labeled leukocytes. Deysine, Robinson, and Wilder (*2*) also successfully delineated rabbit abscesses by a similar method. Several problems may arise when this technique is applied to detecting human abscesses. First, because the leukocytic pool for potential migration to inflammatory sites is enormous, a large population of leukocytes will have to be tagged to visualize an abscess (*3,4*). Second, reported studies have shown that the efficiency of ^{51}Cr tag to leukocytes is highly variable (*1,2,5,6*), complicating the problem of tagging a very large population of leukocytes. Finally, present techniques for isolating leukocytes are probably too complex for many clinical laboratories of nuclear medicine.

As a partial solution to these problems, we have investigated techniques for obtaining concentrates of viable human phagocytes with a minimum of equipment and technical complexity, and we have assayed several variables in obtaining a consistent and efficient white cell tag of ^{51}Cr.

METHODS

Phagocytes concentration. Two to 300 ml of venous blood were drawn from volunteers into 50-ml plastic syringes wetted with 1:1,000 aqueous heparin. The whole blood is diluted in four volumes of 0.8% ammonium chloride (NH_4Cl) in 250-ml centrifuge cups and allowed to hemolyze at room temperature for 15 min. This mixture is centrifuged at 500 G for 15 min at room temperature after which the supernate is decanted. The 0.87% NH_4Cl wash is repeated with similar volumes and a 5-min incubation period at room temperature. The cellular suspension is again centrifuged at 500 G for 10 min, and the supernate is discarded. Most remaining intact erythrocytes are "floated off" with modified Ringer's solution (pH 7.4, 200 mg% glucose) by gentle agitation. Divalent cations should be avoided as they cause leukocyte clumping.

The resultant leukocyte button (including polymorphonuclear leukocytes and monocytes) is finally freed of the remaining erythrocytes by hypotonic shock which is produced with 60-ml distilled water for 20 sec. This shock is then rapidly reversed by the addition of 20 ml 3.6% sodium chloride (NaCl) (*7*). Ringer's solution is added to the leukocyte suspension which is again centrifuged. The concentrated

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leukocyte button is transferred by pipette to a 10-ml plastic tube after being suspended in the desired volume of Ringer's solution for isotope labeling. The leukocyte suspension is agitated with the Vortex-Geni (Scientific Industries Co., Springfield, Mass.) for 10 sec to insure leukocyte distribution. All leukocyte suspensions are washed twice with 0.85% NaCl and centrifuged before measurements of radioactivity. All solutions and equipment are sterile and pyrogen free.

Isotope labeling. Hexavalent, carrier-free ^{51}Cr as Na_2CrO_4 was obtained from Mallinckrodt Nuclear (specific activity 53.5 mCi/μg). Trivalent ^{51}Cr was produced from hexavalent chromium by reduction with 1.0 ml ascorbic acid. All samples were appropriately diluted and counted in a Nuclear-Chicago well counter with a spectrometer baseline at 290 keV and a 50-keV window.

Several variables were investigated with at least 2×10^7 phagocytes except when indicated. Mixing techniques during incubation included rotating wheel (20 rpm), agitation by vortexing every 5 min, or stationary. Other variables evaluated in isotope labeling were pH, length of leukocyte and ^{51}Cr incubation, temperature, concentration of ^{51}Cr, enhancement of phagocytosis by latex particles (Bacto-Latex, Difco Laboratories, Inc., Detroit, Mich.), chemical valence of ^{51}Cr, and volume of incubation mixture (phagocyte concentration).

The affinity of the leukocyte tag was evaluated by three washings with 10 ml of 0.85% NaCl. Localization of the label was determined by fractionation according to the method of Cohn and Hirsch (*8*). Several fractions were isolated by centrifugation at 4°C in 0.34 *M* sucrose (buffered to pH 7.4 with sodium bicarbonate) in a Beckman centrifuge (#L4 Beckman Instruments Corp., Fullerton, Calif.).

RESULTS

Leukocyte concentrates. The number of leukocytes contained in each preparation (70–80% phagocytes) varied with the patient's circulating white blood cell count. Some $1–6 \times 10^8$ phagocytes were consistently obtained from 300-ml venous blood of a single donor. Microscopic examination by counting chamber and Wright's stain generally revealed less than 0.2% erythrocyte contamination.

Our separation technique provided within 1 hr highly purified concentrates of viable phagocytes which appeared to be physiologically competent in vitro. Leukocytes in these concentrates ingested latex particles and bacteria normally by Gram stain techniques, resisted staining by 0.2% trypan blue (99% viability), reduced nitroblue tetrazolium dye in a normal manner (*9*), showed normal lymphocyte blastogenic transformation (*10*), and had normal bactericidal activities (*11*). These in vitro tests of leukocyte function are excellent indicators of cell viability (*12,13*) and, in general, exceed those studies of viability which were performed in other ^{51}Cr leukocyte investigations (*1–6*).

Isotope tagging. Several variables were examined to determine an optimal tagging technique. In general, the tagging efficiency increased from our initial experiments as we gained experience with the isolation techniques. Chromium-51 tagging was not accompanied by abnormal leukocyte clumping as determined by light microscopy. This has been a complication in many leukocyte investigations.

Valence of chromium: The leukocyte tagging of ^{51}Cr is critically dependent on the valence of chromium. Trivalent ^{51}Cr tagging was 0.3 and 1.9% in two experiments compared with 6.8 and 10.3% with hexavalent ^{51}Cr (pH 7.4, rotating for 30 min at 37°C, ^{51} Cr dose of 100 μCi). Hexavalent ^{51}Cr was subsequently used in all further experiments.

Temperature of incubation: White cells were incubated without agitation (stationary) at three temperatures, 20°C, 30°C, and 37°C for 10, 30, and 60 min. No statistically significant differences were noted due to temperature (Student's t-test) (Table 1). This is in contrast to the studies of McMillan and Scott which showed that labeling was temperature related (*6*).

pH of incubation mixture: White cells incubated on a revolving wheel in phosphate buffers with pHs of 5.6, 7.4, and 8.0 gave tagging percentages of 17.9, 36.7, and 20.2%, respectively. Clearly the tagging reaction was pH dependent, and pH 7.4 was used in all other experiments.

Duration of incubation: Phagocytes were incubated for 10, 30, and 60 min in stationary suspensions. As seen in Table 1, tagging was exponential with a moderate amount of ^{51}Cr incorporation by 10 min. We found no statistical differences in percentages of tagging between the 30- and 60-min interval of incubation (Student's t-test, 0.10).

Amount of ^{51}Cr: Aliquots from one leukocyte suspension were incubated with varying amounts of isotope which are expressed in three different ways in Table 2. The percentage of tag decreased while the absolute amount of chromium tagged increased from concentrations of 50–200 μCi of ^{51}Cr. McMillan and Scott (*6*) found that incorporation of ^{51}Cr was directly proportioned to ^{51}Cr concentration within the range 7.5–30 μCi/10^8 leukocytes and Hersey (*14*) used 50–100 μCi/ml ($20–50 \times 10^6$ cells) in lymphocyte labeling experiments. This indicates that the binding sites of ^{51}Cr are not saturated at lower concentrations of ^{51}Cr as the tag per 10^6

TABLE 1. INFLUENCE OF TEMPERATURE ON ^{51}Cr TAGGING (% ^{51}Cr TAG)*

Donor No	Incubation time (min)	Temperature (°C) 22	30	37
1	10	26.6	42.5	20.9
2	30	27.5		23.4
3	30	41.1	42.5	38.3
4	60	42.8	42.4	39.8
5	60	32.1		27.5
6	10			26.6
	30			27.5
7	10			20.9
	30			23.4
8	30		38.3	41.1
	60		39.8	42.8
9	30		35.6	42.5
	60		40.9	42.2

* All incubations were stationary.

TABLE 2. RELATIONSHIP OF DOSE ^{51}Cr TO AMOUNT TAGGED*

Amount of ^{51}Cr incubated μCi (μg)	Percentage tag	Absolute amount of Cr tagged μCi (μg)	Amount of Cr_6 tagged/ 10 white cells/ml solution (ln μCi)
50 (1.0)	42.1	21.1 (0.40)	0.31
100 (2.0)	35.4	35.4 (0.66)	0.45
200 (4.0)	27.8	55.6 (1.04)	0.81

* Specific activity 53.5 μCi/μg.

white cells was proportionate to the ^{51}Cr concentration.

Latex particles: The influence of phagocytosis on ^{51}Cr tagging was evaluated with latex particles. As seen in Table 3, the percentages of ^{51}Cr tagging were lower when latex particles were in the ^{51}Cr and leukocyte suspensions than when the same sample contained no latex. These differences, however, were not statistically significant. Thus ^{51}Cr incorporation is not dependent on ingestion of particles from the surrounding media in which the isotope is in solution.

Tagging and motion of the incubation mixture: The percentages of tag in two experiments when the incubation mixture was stationary were 14.9 and 13.6% compared with 16.1 and 16.0% for a rotating mixture. Therefore, ^{51}Cr tagging to phagocytes was rheologically independent.

Leukocyte concentration in ^{51}Cr incubation mixture: The concentration of white cells influences the amount of ^{51}Cr which is incorporated. As seen in Table 4, when the amount of chromium tagged to leukocytes was expressed in terms of the number of white cells per milliliter incubating solution, the efficiency of tagging was directly proportional to the concentrations of white cells. However, if the white cell concentration exceeded 14×10^8 cells/ml of incubating solution, then the total amount of ^{51}Cr incorporation decreased.

Affinity and location of tag: To ascertain the tenacity of the chromium tag, four preparations were subjected to multiple saline washes. The centrifuged cell buttons were then recounted. Consistently less than 10% of the total chromium tag was removed by three consecutive saline washes. While the cell counts decreased 50%, probably due to lysis, once ^{51}Cr is incorporated into the leukocyte, elution by washing is an unlikely event.

In four human phagocyte preparations lysed by freezing, twice as much tag was counted in the debris fraction as in the supernate. This ^{51}Cr tag to leukocytes is probably related to some constituent of cell membrane and/or nuclei since the debris fraction of leukocyte homogenates contains these components.

DISCUSSION

Several physiological parameters are important in obtaining a consistently high chromium tag of leukocytes. Tagging occurs exponentially up to 30 min of incubation after which time further increases are disproportionately slight. Secondly, the incorporation of ^{51}Cr by leukocytes is not related to mixing. Thus, our results agree with others (*6*) that adequate tagging occurs during 30–45-min incubation without constant stirring.

TABLE 3. EFFECT OF LATEX PARTICLES ON TAGGING (% TAG)

	50 μCi	100 μCi	200 μCi
With latex particles	37.9	25.7	21.9
Without latex particles	42.1	35.4	27.8
Student's t-test	0.40	0.35	0.20
		p value	

TABLE 4. EFFICIENCY OF TAGGING WITH VARYING NUMBERS OF WHITE CELLS*

10^7 WBC/ml	μCi ^{51}Cr tagged/ml
6.4	9.9
6.6	11.7
12.8	20.0
32.0	21.4
140.0	11.6
280.0	2.6

* 50 μCi incubated in all tubes, stationary, 30 min duration.

No statistical differences in tagging efficiency were found when the leukocyte suspensions were incubated at room temperature (22°C), 30°C, or 37°C. McMillan and Scott did find some difference in tagging between 22°C and 37°C. Our results also differ from these latter authors in that we found the pH of the leukocyte suspension affected ^{51}Cr labeling. While red cells are efficiently tagged with ^{51}Cr in an environment of acid citrate dextrose (*15,16*), platelets incorporate ^{51}Cr most optimally at a physiologic pH, and much less with pH deviations from this range (*16*). Consideration of pH raises the importance of salt buffers in incubating solutions. Phagocytes can generate significant amounts of lactic acid during incubation (*12*). Therefore, we have routinely used a modified Ringer's salt solution (pH 7.45, 200 mg% glucose) as part of the incubating medium.

Other factors in the tagging process are the valence and amount of chromium used. It is well recognized that the valence of the chromium ion is critical in tagging (*1,2,5,6,18,19*). In the trivalent form chromium binds readily to ambient proteins and apparently does not enter erythrocytes, leukocytes, or intestinal epithelium (*15–20*). However, hexavalent ^{51}Cr readily enters red cells and intestinal epithelial cells (*15,16,20*). Our results similarly indicate that the hexavalent state of chromium is crucial for tagging to leukocytes.

The amount of isotope incubated with isolated leukocytes is also an important consideration when attempting to visualize an abscess isotopically. By analogy with thyroidal ^{131}I uptake, we estimate that 1–3 μCi of ^{51}Cr need to be within or near an abscess to obtain a satisfactory image. This amount of ^{51}Cr can be tagged to leukocytes using our simplified techniques. Our data indicate that 50 μCi of ^{51}Cr (or 1.0 μg chromium in the material used in this study) can be labeled to leukocytes when a 200-μCi dose of ^{51}Cr (4 μg chromium) is incubated with 1–6 $\times$ 10^8 cells (i.e., 0.81 μCi per 10^6 leukocytes). Although the amount of ^{51}Cr was directly proportional to the concentration of ^{51}Cr in the incubation medium, the percentage of tagging efficiency decreased after 50 μCi. Furthermore, the tagging efficiency decreased with either very low (i.e., 6.4 $\times$ 10^7 WBC/ml) or very high white cell concentrates (i.e., 28 $\times$ 10^8 cells/ml). This implies a saturation of ^{51}Cr binding sites and also a direct inhibition of these binding sites by a large number of leukocytes which may clump and obscure potential ^{51}Cr binding sites. Ideally, these effects can be minimized if 100–200 μCi of ^{51}Cr (2–4 μg chromium) are used to label 10 $\times$ 10^8 cells/ml of incubation media.

The precise mechanism by which ^{51}Cr binds to the leukocyte is unknown. In erythrocytes, chromium has a high affinity for B-polypeptide chains of hemoglobin (*21*), whereby 90% of ^{51}Cr is incorporated within 15–20 min (*16*). Some preliminary evidence indicates that ^{51}Cr enters the leukocyte by transport mechanisms (*18*) and then binds to intracellular proteins (*19,22*). Our inability to elute ^{51}Cr from tagged leukocytes with serial washings has been noted by other investigators (*5,6,23*) and may indicate that some type of physiochemical binding occurs in the labeling process.

The location of the ^{51}Cr in the white cell is also unknown. The ^{51}Cr leukocytes (80% polymorphonuclear cells) were homogenized, and the various cell fractions were separated by differential centrifugation in 0.34 *M* sucrose to preserve subcellular elements such as lysosomes. Approximately two thirds of the radioactivity was confined to the "debris fraction" which contains cell membranes, and nuclei phagocytes. An external tagging site is suggested. Phagocytes which were ingesting latex particles bound less ^{51}Cr than nonphagocytizing leukocytes. This ingestive phase is usually noted for the de nova synthesis of lipids and alterations in cellular membranes.

Even if some of these physiological parameters are taken into consideration, a disparity of results are recorded in the literature in the percentages of ^{51}Cr tagged to leukocytes. Leukocytes clearly do not have a great affinity for ^{51}Cr as occurs with erythrocytes. Chromate-51 tags of 1–3% and higher (80%) are commonly reported for a variety of leukocyte preparations (*1,2,5,6*). The considerable variability in labeling of leukocytes probably arises from the quality and quantity of leukocytes which are used.

Frequently, as McMillan and Scott have indicated (*6*), 60–75% of leukocytes from a donor are lost in the concentration methods whereas the techniques described here consistently isolated 70–80% of donated leukocytes. By several tests, these isolated tests showed the cells were viable and physiologically intact. These tests included the exclusion of trypan blue dyes, a criterion demonstrated by Rogentine and Plocinik (*24*) to correlate well with leukocyte viability in tissue typing studies. Other tests of both lymphocyte (DNA synthesis) and granulocyte viability (phagocytosis, migration, nitroblue tetrazolium dye reduction) were within the normal limits for cells isolated by more classical techniques.

In the past, erythrocyte contamination of leukocyte preparations has been a limiting problem. The techniques described here eliminate this problem by lysing more than 99% of all erythrocytes with serial ammonium chloride washes and one hypotonic shock lysis.

Because we were interested in phagocytes rather than the lymphoid series, these cell concentrates were produced so that 70–80% of the cells were phagocytes. These could be isolated within 1 hr by the present techniques. This time limit is important because these phagocytes deteriorate in viability over the second to fourth hour in vitro (*13*). Thus, in human leukokinetic studies, the initial recovery of ^{51}Cr-tagged leukocytes was less than when DF^{32}P was used (*6,25*). Cell viability may have been reduced as the former isotope-labeling techniques employ measures to separate and lyse erythrocytes.

Several investigators have shown that there is a relative order of avidity for ^{51}Cr between the cellular elements of blood (*6,14,26*). It appears that large lymphocytes and monocytes are labeled with ^{51}Cr to a greater degree than polymorphonuclear leukocytes (*26*). Therefore, investigators of leukokinetics in various disease states should be aware that leukocyte concentrates from whole blood will produce a variable ^{51}Cr tag unless more definitive separation techniques are used.

In conclusion, these studies have shown that large quantities of viable erythrocyte-free phagocytes can be obtained by simple methods. These leukocytes were then used to define means by which a more efficient ^{51}Cr tag could be obtained. The site of the ^{51}Cr tag is unknown. Whether these techniques can be applied to the detection of human abscesses, as others have shown in rabbits, warrants further investigation.

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