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Human Monocyte Chemotaxis: A Quantitative *in Vivo* Technique

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This report describes a new quantitative technique for evaluating monocyte chemotaxis to a site of superficial epidermal abrasion. Micro-acrylic chambers containing 50% Zymosan activated autologous serum were separated from a 5-mm diameter epidermal abrasion by 2 Nucleopore filters which entrapped migrating monocytes but allowed free neutrophil migration. Monocytes were specifically identified by alpha napthyl acetate esterase activity. Monocytes accumulated within the filters by 4 hr and maximized at 16 and 20 hr.

This technique is superior to previous skin chamber techniques in the high yield of monocytes and in specific histochemical identification of monocytes. In contrast to the Rebuck window, it does not generate attractants and has greater reproducibility.

This technique will be useful in the study of diseases characterized by monocytic infiltrates, in contrasting the function of peripheral blood monocytes to those available in the skin, and in testing the effects of drugs, immunodeficiency and infection on monocyte function *in vivo*.

Quantitation of the ability of leukocytes to reach the skin in response to abrasion or specific attractants has been an important means of studying the availability and responsiveness of inflammatory cells *in vivo*. In 1955 Rebuck and Crowley described a technique of measuring adherence of leukocytes to a glass coverslip applied to an abrasion to the papillary dermis [1]. In subsequent improvements of the Rebuck technique, quantitation was somewhat improved, new means of abrasion and attractants were introduced, and specific staining allowed more accurate cell definition [2–3]. Skin chamber techniques were introduced but the proportion of monocytes which entered the fluid phase of the chamber was usually never greater than 10%, even late in the inflammatory response [4–7]. Otani and Hugli recently reported a microchamber technique using Nucleopore filters to semiquantitatively identify neutrophils and monocytes in inflammatory infiltrates [8]. The identification of monocytes in these techniques has been quite variable and dependent on means of abrasion, attractants, means of specifically differentiating monocytes from other inflammatory cells, and the time of sampling. In this paper we present a new quantitative method for identifying and quantitating monocyte migration to specific attractants applied over superficial epidermal lesions.

METHODS

Subjects

Thirty-nine normal healthy volunteers were used in various stages of the development of this technique. Subjects with active clinical infections or a history of post-traumatic hyperpigmentation or keloid formation were excluded. Use of subjects in this experiment was authorized by the Human Research Committee of the University of Colorado.

Skin Abrasion

Skin was shaved and cleansed with Betadine and alcohol and allowed to dry before abrasion. Two different techniques of abrasion were compared.

Tape Abrasion: Blenderm (3M Company, St. Paul, Minnesota) tape was repeatedly applied to a 5-mm circular template on the skin until a uniform glistening surface without macroscopic bleeding was produced. Transient erythema and stinging were common. Six such abrasions could be performed in 15 to 20 min.

Eraser Abrasion: An electric ink eraser (Paos Auto Eraser, Hong Kong) was applied to the 5-mm diameter template for short bursts with rotation to minimize irregularity. Uniform glistening abrasions were the object; abrasions with gross bleeding were not used. Complications: Pain and local heating of the skin occurred.

Attractants

Zymosan was produced by reducing, alkylating and heating yeast with modification of the method of Hadding [9] described by Lachmann, Hobart, and Aston [10]. One pound of Baker's yeast is suspended in 2 liters phosphate buffered saline (PBS) and autoclaved for 30 min at 120°C. The yeast is centrifuged and washed in PBS until the supernatant is clear. The yeast is then resuspended in 250 ml PBS and made to 0.1 M with mercaptoethanol and incubated at 37°C for 2 hr with stirring. The mercaptoethanol is removed from the yeast by wash and centrifugation in saline. The yeast is suspended in 500 ml 0.02 M iodoacetamide in 0.85% NaCl containing 20% by value of 0.2 m phosphate buffer at pH 7.2 and stored at room temperature for 2 hr. Occasional check of pH to keep it at 7.2 is useful but usually not necessary. The yeast is centrifuged and washed in PBS until the supernatant is clear and is finally resuspended in 1 liter of complement-free Veronal buffered saline pH 7.2 containing optimum Mg^{++} and Ca^{+} and sodium azide and is stored at 4°C. Two-tenths of a milliliter of this preparation was added per cc of serum and mixed on a tilt table for 45 min at 37°C. The particulate matter was separated by centrifugation at 2000 g for 10 min. This Zymosan-activated autologous serum (ZAS) was diluted at various concentrations with Hank's balanced salt solution (HBSS). Clotted autologous serum diluted 50:50 with HBSS and undiluted HBSS were also contrasted with diluted ZAS as attractants.

Chambers

Small plexiglass cylinders of 6-mm internal diameter and 7-mm height were closed on one end with a rubber cap and used as skin chambers. Chambers could be autoclaved or chemically sterilized and the caps replaced after each experiment.

Chambers were filled with 0.2 ml of attractant and Nucleopore filters (Nucleopore Company, Pleasanton, California) cut to an 8-mm diameter were placed over the fluid-filled chambers. The chambers were then inverted over the 5-mm diameter circular abrasion on the skin of the subject. Chambers were held in place by 2 pieces of tape and the entire array of 6 chambers was stabilized with an elastic bandage. No adhesive was used to attach filters to the chambers; the light pressure of the tape and bandage formed a tight seal. Figure 1 illustrates this arrangement. Two Nucleopore filters were used in each chamber: A 5 μ pore filter on the skin side and a 3 μ pore filter on the "chamber side."

Harvesting Filters

At designated times the chambers were removed, the filters were separated and scraping of the "chamber side" of the 3 μ filter was done to remove all cells which had crossed the 5 μ and the 3 μ filters. The fluid from the chambers was also saved for hemocytometer quantitation and differential cell counts.

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Abbreviations:
HBSS: Hanks balanced salt solution
PBS: phosphate buffered saline
ZAS: zymosan-activated autologous serum

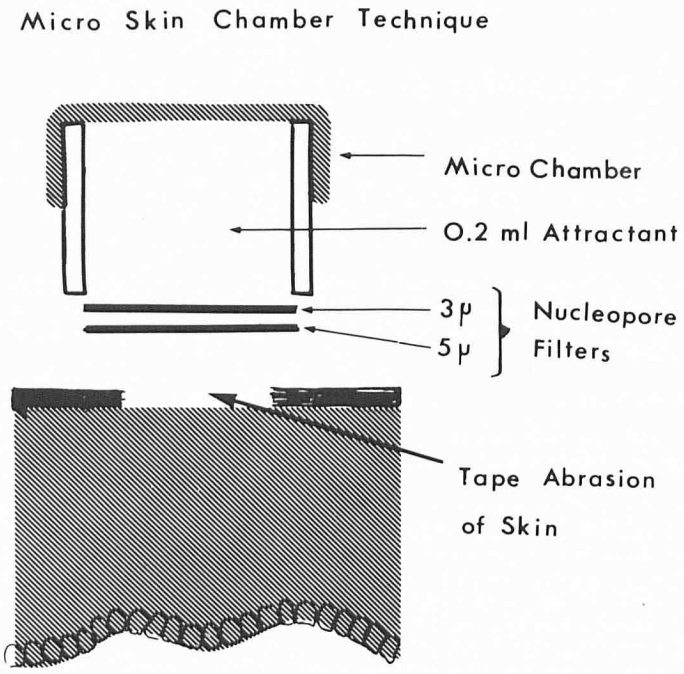

FIG 1. Micro skin chambers containing attractant are separated from epidermal abrasion site by a sandwich of Nucleopore filters.

The 3 μ and 5 μ filters were then separated, fixed, stained and mounted for microscopic quantitation. Staining was initially done with hematoxylin, but eventually staining for alpha napthyl-acetate esterase ("nonspecific esterase") activity was adopted as a convenient and accurate means of visually differentiating monocytes from lymphocytes and neutrophils [11]. In this technique filters were removed from the skin, separated and immediately fixed in buffered formalin, incubated in hexozcetized para rosaniline alpha napthyl acetate solution for 75 min and washed in distilled water 3 times. Counterstaining was with methyl green for 3 min. Filters were then cleaned in distilled water, dried and mounted. Monocytes stained with many small bright red granules, lymphocytes often with a single eccentric large red granule and neutrophils stained only with the counterstain.

Counting Filters

Monocytes and neutrophils in ten oil power (100×) fields of both the 3 μ and 5 μ filters were counted. Cells on either side of each filter or in the pores of the filters were counted. The sum for both cell types in these 10 fields was then multiplied by 142 to express the number of cells in the entire circular 5-mm abrasion. This number was derived by comparing the relative area in ten high power grids to the area of the 5-mm circular abrasion. The density of cells across the abrasion was relatively uniform and the 10 fields selected were at standard locations in the field and not selected because of relative cellular density. The cells on either side of the 5 μ filter, on the "skin" side of the 3 μ filter, or in the pores of either filter will be referred to as "within" the filters, noting attachment to or passage into the filter sandwich.

The scraping of the "chamber side" of the 3 μ filter and the aspirate of the chamber were combined, cell numbers quantitated and a cell differential performed. This is referred to as cells "crossing" the 3 μ filter.

Scraping the "chamber" side of the 3 μ filter was quite effective in removing cells crossing the 3 μ filter and the cells counted on the 3 μ filter after scraping were usually in the pores or on the "skin side" of the filter.

Statistics

Significance was determined by Student *t*-test.

RESULTS

Filter Combinations, Staining and Separation of Filters

Sixteen subjects wore various combinations of 6 chambers to qualitatively and quantitatively identify the best combinations of chamber size, filter number and pore size and methods of separating, fixing and mounting filters. Figure 2 illustrates the most useful filter combination—a 5 μ pore filter on the "skin side" and a 3 μ pore filter on the "chamber side." Between 12 and 20 hr 52% of the cells "within" the filters were monocytes. At these times less than 5% of the cells "crossing" the 3 μ filter were monocytes.

Nonspecific esterase staining was far superior to hematoxylin staining; at 16 hr the cells absolutely identified as monocytes were 3 times greater when specific histochemical staining was contrasted to hematoxylin staining and identification by cell morphology ($69.6 \pm 14.2 \times 10^3$ and $19.7 \pm 8.4 \times 10^3$ cells per chamber, respectively $p < .001$).

"Nonspecific" esterase staining also allowed absolute differentiation of lymphocytes and monocytes. Although lymphocytes were occasionally seen at the skin surface of the 5 μ pore filter, they were found very infrequently to have migrated into the filters. Biopsies of tissue sites containing skin chambers showed massive monocyte and neutrophil migration into the dermis and epidermis, but only dermal perivascular lymphocyte accumulation. Red blood cells were frequently seen on the skin side of the filters, were easily identified but were not counted in cell differentials.

Filters were also stained after separation and without separation to determine if cells were lost when separating the filters. The cell counts were uniformly higher on separated filters, due largely to better fixation, staining and visualization of cells.

Eraser Versus Tape Abrasion

In 7 subjects, migration to 50% ZAS and HBSS was contrasted over eraser and tape-abraded skin. Table I shows that the number of monocytes migrating into control chambers

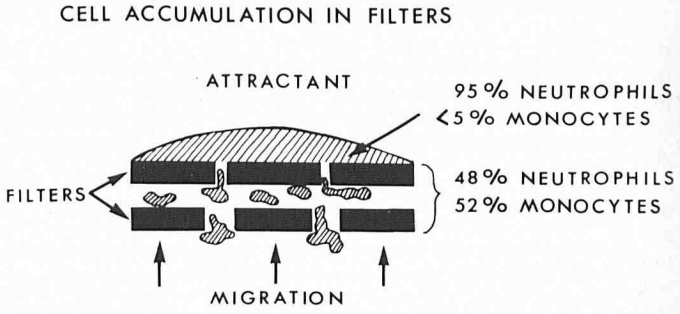

FIG 2. Schematic accumulation of cell accumulation in the filter sandwich. The "chamber side" filter has 3 μ pores and the "skin side" filter has 5 μ pores. Cells "crossing" both filters accumulate on the chamber surface of the 3 μ filter. The cells which cannot cross the 3 μ pores are "within" the filters: in pores, between the 3 μ and 5 μ filters, and on the skin side of the 5 μ filter. Between 12 and 20 hr > 50% of the cells within the filters are monocytes and < 5% of the cells crossing the 3 μ filter are monocytes.

TABLE I. *Eraser versus tape abrasion*[a]

	Cells × 10^3		
	Tape	Eraser	
Control (HBSS[b])	.39 (±.39)	7.34 (±2.64)	p = .01
Attractant (50% ZAS[c])	21.1 (±3.5)	31.9 (±3.65)	
Chemotaxis (attractant-control)	20.7 (±3.4)	24.5 (±3.5)	

[a] Monocyte migration in thousands of cells per chamber (mean ± standard error of the mean) in 7 subjects comparing the results over eraser and tape abraded skin to HBSS and 50% ZAS as attractants. Chemotaxis is expressed as migration to specific attractant minus migration to medium as attractant (HBSS). There is nearly a 20-fold increase in background attraction when eraser rather than tape is used to produce epidermal abrasion (p = .01). Chemotaxis, on the other hand, is not significantly different.

[b] HBSS = Hanks' balanced salt solution.

[c] ZAS = zymosan-activated autologous serum.

(HBSS as "attractant") over tape-abraded skin was nearly 20 times less than migration over eraser-abraded skin (p = .01). Chemotaxis, defined as migration to ZAS attractant minus migration to HBSS control, was not significantly different using the 2 techniques of abrasion. The cell counts were done on hematoxylin-stained filters; subsequent controls with nonspecific esterase staining showed persistently low control migration over tape-abraded skin and we chose this as our standard means of abrasion because of this low baseline migration.

Attractant Effectiveness

In 4 subjects, various dilutions of attractant (1%, 10%, 50%, 100% ZAS in HBSS) were contrasted to medium (HBSS) and clotted serum as attractants of monocytes *in vivo*. The effectiveness of 100% and 50% ZAS were comparable (59.6 ± 6.6 and 59.6 ± 9.8 thousand cells per chamber respectively) and were significantly greater than the effectiveness of 10% ZAS (35.6 ± 7.6 thousand cells per chamber p = .02) or 1% ZAS (4.6 ± 2.9 thousand cells per chamber p = .001). Both clotted serum and HBSS had minimal effectiveness as monocyte attractants in this *in vivo* system. Fifty percent ZAS was subsequently selected as our standard attractant.

Time Course of Migration

Six subjects wore 12 chambers containing 50% ZAS attractant for 24 hr. Duplicate chambers were harvested at 4, 8, 12, 16, 20, and 24 hr from each subject. Filters were fixed and stained for nonspecific esterase activity and the scrapings of the "chamber side" of the 3 μ filters and chamber aspirates were quantitated and differentials obtained.

Table II shows the cells "within" the filters (i.e., not crossing the 3 μ filter) at the times of harvest. Monocytes appeared by 4 hr and the total number of monocytes within the filters maximized at 16 and 20 hr, falling slightly at 24 hr. The numbers of neutrophils progressing increased over the course of the study. By 12 hr a consistent proportion of the cells within the filters were monocytes (52.0 to 54.8%).

Table III shows the numbers of monocytes and neutrophils "crossing" the 3 μ filter at the index times. At all times neutrophils were the vast majority of cells. At 24 hr the number and relative percentage of monocytes increased markedly; before 24 hr less than 5% of the cells crossing the 3 μ filter were monocytes.

TABLE II. *Cells within the filters (× 10³)*[a]

Time, hr	Monocytes	Neutrophils	% Monocytes
			Total
4	3.37 ± 1.5	17.3 ± 6.7	16.3
8	24.9 ± 8.7	36.2 ± 17.9	40.7
12	52.8 ± 11.2	47.1 ± 10.9	52.9
16	68.8 ± 15	62.2 ± 23.8	52.5
20	69.6 ± 14	57.4 ± 18.6	52.0
24	59.6 ± 9.8	58.5 ± 12.1	54.8

[a] The numbers of monocytes and neutrophils in thousands of cells retained "within" the filter sandwich (mean ± SEM) is expressed at each index time. The percentages of cells within the filters which are monocytes at each time is also indicated. Monocyte accumulation within the filters maximizes at 16 and 20 hr.

TABLE III. *Cells crossing 3 μ filter (× 10³)*[a]

Time, hr	Monocytes	Neutrophils	% Monocytes
			Total
4	—	74 ± 32	
8	13.7 ± 8.2	458 ± 200	2.9
12	35.9 ± 18	1176 ± 661	3.0
16	42.4 ± 15.0	1231 ± 300	3.4
20	59.5± 31.1	1616 ± 341	3.6
24	187.0 ± 108	2437 ± 1108	7.6

[a] Monocytes and neutrophils crossing the 3 μ filter in thousands of cells per chamber (mean ± SEM) is shown at each time of harvest. The percentage of total cells crossing the filters which are monocytes is also shown. Up to 20 hr, consistently less than 4% of the cells crossing the 3 μ filter are monocytes.

TABLE IV. *Comparison of monocytes within filters versus monocytes across 3 μ filter*[a]

Hour	Within filters (× 10³)	Across 3 μ Filter (× 10³)
4	3.37 ± 1.5	—
8	24.9 ± 8.7	13.7 ± 8.2
12	52.8 ± 11.2	35.9 ± 18
16	68.8 ± 15.0	42.4 ± 15
20	69.6 ± 14.0	59.5 ± 31
24	59.6 ± 9.8	187 ± 108

[a] This shows the numbers of monocytes retained within the filters and crossing the 3 μ filter in thousands of cells per chamber (mean ± SEM) at the index times. Note the persistent increase in monocytes crossing the 3 μ filter and the sudden increase at 24 hr, accompanied by a decrease in monocytes within the filters at that time.

Table IV is a direct comparison of the monocytes within the filters versus those crossing the 3 μ filter at the index times. The numbers of monocytes within the filters were greater than those crossing the 3 μ filter until 24 hr, when there was a substantial increase in cells crossing the filter sandwich. This suggests that by 24 hr the monocytes traverse the 3 μ filter in greater numbers than those migrating into the 5 μ and 3 μ filter sandwich. After 12 hr the standard errors of the numbers of monocytes crossing the 3 μ filter were usually greater than the errors of the cells counted with the filters. In addition we feel that the aspirate and scraping data used to derive cells "crossing" the 3 μ filter are particularly prone to variability caused by leakage, retention of fluid on the skin, and difficulties in getting consistent monocyte proportion in cytocentrifuge preparations from the aspirates and scrapings. For these reasons, we feel that quantitation of monocytes "within" the filters at 16 or 20 hr is the most consistent technique to measure maximal monocyte migration. Measurement of neutrophil migration, however, must take into consideration the mass of neutrophils crossing the 3 μ filter. We feel that a scraping of the chamber side of the 3 μ filter performed at 12 to 20 hr will produce large numbers of neutrophils with minimal monocyte contamination.

Thus both neutrophil and monocyte chemotaxis can be measured concurrently and separated successfully by using specific cellular stains, filter trapping of monocytes, and sampling of different cellular populations—those crossing the 3 μ pore filter, and those retained "within" the 3 μ and 5 μ pore sandwich.

DISCUSSION

Leukocyte migration into the skin in response to abrasion or specific attractants is a complex process dependent on available peripheral blood, marginal pool and tissue leukocytes; on vascular permeability changes; on the ability to traverse numerous physiologic cellular and basement membrane barriers; on the generation of endogeneous attractants by abrasion and the addition of specific attractants; and on the ability to attach to collecting coverslips or filters or detach and float into a collecting chamber. Our technique has several advantages over other collection techniques. First our method of abrasion produces a controlled superficial lesion [12] with little generation of endogenous attractants such as C5a [13] or "leukoegresin" [14]. Avoiding frequent removal of chambers also keeps generation of endogenous attractants low. Because background migration is so low, this method is a useful means for critically evaluating relative attractant specificity for monocytes *in vivo*. Second, migration into filters does not require cells to detach from tissue surfaces and enter a fluid phase to be harvested. Chamber techniques traditionally harvest less than 10% monocytes even with extended periods of incubation [6,7], while in the Rebuck skin window the monocyte becomes the predominant cell after 12 hr [1,3]. In our technique, large proportions of mononuclear cells can be harvested as with the Rebuck window, but large

volumes of specific attractants as used in skin chamber methods can be employed [6]. The filters which we use also represent a better surface than glass coverslips for collecting cells; use of the latter may select for monocytes late in the inflammatory response because of their superior glass adherent properties. Third, the nonspecific esterase stain provides a means of accurately identifying monocytes in a population of cells distorted by migration through small pores, spreading over various levels of focus, and lying super-imposed or in masses on filters. Fourth, by using multiple small chambers, subjects can easily serve as their own controls and duplicate chambers can control for variability. The small chambers are also more comfortable and produce less dyspigmentation than the large chambers. Simultaneous quantitation of neutrophil and monocyte migration can be performed.

In experiments where aspirates and chamber-side filter scrapings were compared to cells "within" the filters, we found that the cells "within" the filter, if quantitated at 16 or 20 hr, represented a less variable measurement of monocyte migration. Hemocytometer counts and differential of cytocentrifuged cell films to determine cell numbers in the fluid phase are quite variable, depending too much on leakage, degree of scraping, differential adherence of monocytes to glass slides for staining, and aspiration of cells from the abraded skin surface when aspirating fluid left on the abrasion site after removal of the chamber.

It is interesting that potent attractants such as 50% ZAS produce significant monocyte chemotaxis *in vivo* while clotted serum, a good neutrophil attractant *in vivo* is quite ineffective. This suggests that quantitatively and qualitatively the neutrophil is much more readily available at sites of epidermal injury than is the monocyte. This fact is certainly supported by the many *in vitro* studies which show that neutrophils pass through smaller openings more quickly than do monocytes. It is obvious that migration into sites of epidermal abrasion *in vivo* requires movement across many physiologic barriers which are more easily passed by neutrophils than monocytes. However, even monocytes can be seen as early as 4 hr at sites of superficial epidermal abrasion.

Another observation is the lack of lymphocytic migration in this system. Even biopsies of sites bearing chambers show little more than mild perivascular lymphocytic involvement. Lymphocytes may require more time, a more persistent chemotactant gradient, or generation of other attractants *in vivo* to demonstrate significant movement to sites of epidermal injury.

The Boyden chamber technique is an important means of evaluating chemotaxis of peripheral blood or tissue exudate monocytes or macrophages in controlled *in vitro* conditions. The technique described in this paper provides a means of measuring monocyte chemotaxis *in vivo* where variables such as vascular permeability, marginal pool and tissue cellular availability, and intact physiologic barriers can affect migration. This technique can be used to contrast the function of peripheral blood cells and those available at sites of skin abrasion, and the effects of drugs, immunodeficiency or infection on these cellular populations. It should also be easily adaptable to harvesting cellular infiltrates over diseases characterized by monocytic infiltrates such as histiocytosis-X, mycosis fungoides, and reticulohistiocytosis.

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