Journal of Immunological Methods, 37 (1980) 39—45

RAPID QUANTITATION OF NEUTROPHIL CHEMOTAXIS: USE OF A POLYVINYLPYRROLIDONE-FREE POLYCARBONATE MEMBRANE IN A MULTIWELL ASSEMBLY

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(Received 3 April 1980, accepted 24 April 1980)

A neutrophil chemotaxis assay was developed which permits rapid, quantitative assessment of migration across a membrane filter. The critical factor in the assay was the use of a 10 μm thick polycarbonate membrane without the usual polyvinylpyrrolidone coating. Migrated neutrophils remain adherent to the uncoated membrane, whereas 20—50% fall off polyvinylpyrrolidone-coated membranes. A major advantage of the method is that neutrophil chemotaxis can be readily quantified, since the migrated cells adhere to the membrane surface and are in one optical plane for counting. A 25 mm × 80 mm membrane sheet was used in a 48-well micro chemotaxis assembly, which requires only 20,000 neutrophils and 25 μl of attractant per assay well. Neutrophil chemotaxis was complete within 10—20 min at 37°C, with 20—30% of the cells migrating to N-formyl-methionyl-leucyl-phenylalanine and 40—50% migrating to complement derived C5a.

INTRODUCTION

Since 1917, a variety of methods have been developed to study neutrophil (PMN) chemotaxis. They include techniques for studying the movement of individual PMNs on slides (Grimes and Barnes, 1973; Zigmond and Hirsch, 1973), migration under agarose (Carpenter, 1963; Clausen, 1971; Cutler, 1974; Nelson et al., 1975), and movement through a membrane filter (Boyden, 1962).

PMN chemotaxis through filters has frequently been used in clinical studies and for examining dose responses of PMNs to various chemoattractants because of the simplicity and quantitative potential of the technique. Two types of filters used are polycarbonate filters, 10 μm in thickness (Horwitz and Garrett, 1971) and cellulose filters, 100—150 μm in thickness (Boyden, 1962). Although cellulosic filters are useful for certain analyses, quantifying the total number of migrating cells is tedious since counts must be done in a series of optical planes through the 100—150 μm thickness of the filter. In contrast, PMNs which have migrated through the thin polycarbonate filters are found in one optical plane and their morphologic appearance is excellent. A serious problem with both types of filters has

been the dropping off of migrated PMNs from the lower filter surface. Methods have been described in which two filters (one cell-permeable and the other cell-impermeable) have been sandwiched to trap migrated cells (Keller et al., 1972; Campbell, 1977). These methods have been successful in trapping migrated cells; however, the manipulation and staining of sandwiched filters have been impractical for routine chemotaxis assays.

Since polycarbonate membranes permit rapid quantitation of chemotactic responses, we examined conditions to improve PMN adherence to the filters. Most commercially available polycarbonate filters are coated with polyvinylpyrrolidone (PVP) to enhance their wettability. We found that a significant and variable percentage of migrating PMNs fell off the lower surface of the PVP-coated filters, so that quantification of responses was impossible. The problem was solved by the use of PVP-free polycarbonate membranes. Horwitz and Garrett used PVP-free polycarbonate membranes in neutrophil chemotaxis experiments in 1971, but the effect of PVP coating was apparently not appreciated at that time. In this report, we compare PVP-coated and uncoated membranes, and describe a new assay which utilizes a 25 mm × 80 mm uncoated polycarbonate membrane in a recently described (Falk et al., 1980) 48-well micro chemotaxis assembly.

MATERIALS AND METHODS

Leukocyte preparation

Human neutrophils were obtained from heparinized blood of healthy donors (10 units of heparin per ml of blood). Blood was sedimented with 5% dextran solution in 50 ml polypropylene tubes at room temperature for 40 min. The leukocyte rich plasma layer was mixed with 6 vol of ice-cold Hanks balanced salt solution (HBSS) and centrifuged for 10 min at 500 × *g*. The cell pellet was resuspended in 5 ml of ice-cold saline and residual erythrocytes were hypotonically lysed (Goldstein et al., 1973). Leukocytes were resuspended and washed twice with 50-ml vols of ice-cold HBSS. Final cell suspensions were prepared with cold HBSS. Suspensions contained 89—98% neutrophils which were greater than 97% viable (assessed by trypan blue dye exclusion).

Chemotactic factors

N-formylmethionyl-leucyl-phenylalanine (FMLP) and N-formylnorleucyl-leucyl-phenylalanine were generously supplied by Dr. Elliott Schiffman (National Institutes of Health). The peptides were dissolved in ethanol and dilutions were made with HBSS. Human serum derived complement component C5a was prepared by modification of the method of Fernandez and Hugli (1976). Briefly, 100 ml of serum were activated by incubation at 37° C with 100 mg of zymosan in the presence of 13 g of ϵ-aminocaproic acid.

After 45 min the serum was cooled in ice and acidified with hydrochloric acid to pH 3.8. The serum was then centrifuged and dialyzed against 0.1 M ammonium formate buffer, pH 5.0, containing 0.1 mM EDTA. The dialyzed solution was added to a column of CM-Sepharose CL-6B (2.6 cm diameter, 100 ml of gel) and eluted with dialysis buffer. The column was then washed with 250 ml of 0.3 M ammonium formate buffer (pH 5.0) and the activity was eluted with 0.5 M buffer. The pooled fractions were concentrated by lyophilization after dialysis, applied to a Sephadex G-100 column and eluted with ammonium formate buffer (0.1 M, pH 5.0). Activity was in the region of 12,000 Daltons. The fractions were pooled, dialyzed against water, lyophilized and reconstituted in 10 ml of phosphate-buffered saline. Aliquots were stored at −20°C.

Chemotaxis assay

Initially, blind well chambers (Neuro Probe, Inc., Bethesda, MD) were used to determine optimum chemotaxis conditions. Bottom wells were filled with 200 μl of chemoattractant; 10 μm thick, 3 μm pore diameter polycarbonate membranes (Nucleopore Corp., Pleasanton, CA) were fixed in place to separate bottom from top wells, and 10^5 neutrophils in 200 μl of HBSS were added to the upper wells. Membranes with or without PVP coating were tested. The only difference in the processing of the two types of filters was the PVP coating step. Blind well chambers were incubated for various time intervals at 37°C in humidified air. In some experiments, 25 mm^2 glass coverslips were placed in the bottom chambers with chemoattractants to collect neutrophils dropping off the filter surface after migration. Filters and coverslips were removed and stained.

After optimal chemotaxis conditions were established, a neutrophil chemotaxis assay was developed with a 48-well micro chemotaxis assembly (multiwell chamber) designed in this laboratory (Falk et al., 1980). To the bottom wells were added 25 μl of chemoattractant. A polycarbonate filter sheet (25 mm × 80 mm) without PVP coating, containing 3-μm holes (Nucleopore Corp., Pleasanton, CA) was placed on top of the wells in the bottom plate. The gasket and top plate were fixed in place and the assembly was incubated for 60 min at 37°C in humidified air (with or without 5% CO_2). After incubation, the top plate, gasket and filter were removed, cells on top of the filter were wiped off and the filter was fixed in methanol for 1—2 min. The filter was air-dried on a glass slide and stained with Diff-Quick (Harleco, Gibbstown, NJ).

Equipment for cell counting consisted of a Leitz microscope with a regulated power supply for precise control of illumination intensity, a VG-9 green filter and an ND 35% neutral density filter in the incident light path, an Optomax Image Analyzer (Optomax Inc., Hollis, NH) equipped with a Chalnicon television camera, and a Hewlett Packard 9815A calculator for printing out cell counts. Filters and coverslips from the blind well chambers

were examined with a 54× oil objective; five fields of cells (a total filter area of 0.26 mm^2) were counted. Migrated cells on the multiwell filter were examined with a 10× objective; two fields (a total filter area of 1 mm^2) were counted.

RESULTS

Development of the new assay

Initial experiments with blind well chemotaxis chambers demonstrated that the PVP coating of polycarbonate filters substantially altered the adherence of PMNs to the filter surface after chemotaxis. Table 1 illustrates the quantitative differences observed between PVP-coated and uncoated filters. When PVP-coated membranes were used, approximately 30% of neutrophils that migrated toward the chemotactic peptide FMLP fell off the filter and were found on the glass coverslip in the bottom well. In contrast, all of the migrating neutrophils remained attached to uncoated filters. The presence or absence of PVP coating on the filters did not significantly alter the total number of migrated PMNs. A number of experiments were performed in which the peptide concentration, neutrophil number and incubation period were varied. It was observed that a substantial but variable number (20—50%) of migrating neutrophils fell off the PVP-coated membranes, irrespective of neutrophil concentration in the top wells or peptide concentration in the bottom wells. When the incubation period was increased (up to 3 h), the numbers of neutrophils dropping off the PVP-coated membranes increased. In contrast, PMNs remained adherent to the uncoated membranes at all cell and peptide concentrations and time periods tested. One factor that decreased neutrophil adhesion to uncoated filters was the presence of 2% bovine serum albumin in the suspension medium. As a result of these findings, subsequent chemotaxis experiments were performed with

TABLE 1

Comparison of neutrophil migration on PVP-coated and uncoated polycarbonate filters.

Assays were performed in blind well chemotaxis chambers with bottom wells containing 25 mm^2 glass coverslips and either HBSS or 10^{-7} M FMLP peptide. Chambers were incubated for 60 min at 37°C. The data are expressed as mean ± 1 S.E.M. from 3 different cell donors, and represent neutrophils per 0.26 mm^2 filter or coverslip surface.

Attractant	PVP-coated filter			Uncoated filter	
	PMNs on filter	PMNs on coverslip	Total PMNs migrating	PMNs on filter	PMNs on coverslip
HBSS (control)	270 ± 55	4 ± 1	274 ± 56	320 ± 20	0 ± 0
FMLP (peptide)	550 ± 170	190 ± 65	740 ± 235	1000 ± 140	2 ± 1

uncoated polycarbonate membranes and HBSS alone as the suspension medium and diluent.

The time course of neutrophil chemotaxis across the polycarbonate membrane is illustrated in Table 2. Chemotaxis was complete within 10—20 min, after which the number of migrated neutrophils did not increase. Incubation periods of 3 and 4 h were also tested to determine whether there might be a subpopulation of neutrophils with much longer response times, but the number of migrated cells did not increase. An incubation period of 60 min was arbitrarily selected for subsequent chemotaxis experiments.

Quantification of neutrophil chemotaxis

Neutrophil chemotaxis to three different chemoattractants was evaluated in the multiwell chemotaxis chamber. The dose response data to FMLP are illustrated in Fig. 1. In tests of PMNs from 5 normal donors, the maximum chemotactic response occurred at a peptide concentration of 10^{-6} M. The optimal response to N-formylnorleucyl-leucyl-phenylalanine was also at 10^{-6} M (data not shown). The response of neutrophils from 3 normal donors to various dilutions of complement derived C5a is shown in Fig. 2. An optimal chemotactic response occurred between relative concentrations of 10^2 and 10^3 M.

Estimates were made of the fraction of neutrophils migrating toward chemoattractants. The data in Figs. 1 and 2 are counts of migrated cells per 1 mm^2 of filter area. Since the total filter area per well is 8 mm^2, multiplication of cell counts by 8 gives the total number of migrated PMNs per assay well. Division by the number of PMNs added to the top well (22,000) gives the fraction migrated. From the data in Figs. 1 and 2, 20—30% of the input PMNs migrated to optimal concentrations of FMLP and 40—50% migrated to C5a.

TABLE 2

Neutrophil migration after various incubation periods.

Assays were performed in blind well chemotaxis chambers with either HBSS or 10^{-7} M FMLP peptide. The data are expressed as the mean ± 1 S.E.M. of triplicate values.

Incubation period (min)	Neutrophils/0.26 mm^2 filter surface	
	HBSS (control)	FMLP (peptide)
3	0	0
5	2 ± 1	152 ± 5
7	36 ± 4	273 ± 10
10	200 ± 90	1390 ± 80
20	420 ± 200	1770 ± 240
120	460 ± 270	1710 ± 160

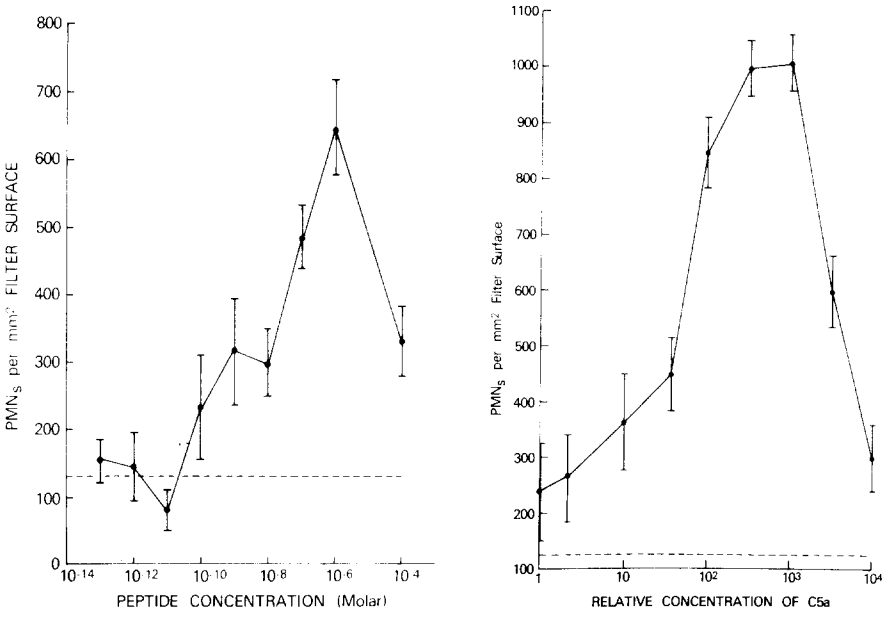

Fig. 1. Dose response of human neutrophils to chemotactic peptide. A multiwell chemotaxis chamber was used to evaluate the PMN dose response to N-formyl-methionyl-leucyl-phenylalanine. Values represent the number of PMNs migrating per 1 mm^2 filter surface as determined with an image analyzer. The error bars represent the standard error of the mean of values from 5 different PMN donors. The broken line indicates PMN response to Hanks balanced salt solution without chemoattractant.

Fig. 2. Dose response of human neutrophils to human C5a. A multiwell chemotaxis chamber was used to evaluate the PMN dose response to human C5a. C5a was prepared as described in the methods section. A relative concentration of 1 means a dilution of $1 : 10^4$ of the C5a stock solution. Migration is expressed as the number of PMNs per 1 mm^2 filter surface counted with an image analyzer. The error bars represent the standard error of the mean of values from 3 different PMN donors. The broken line indicates PMN response to medium alone.

DISCUSSION

In this paper we describe a simple method for quantifying human neutrophil chemotaxis. A critical factor in developing this assay was the selection of membrane filter material. Most polycarbonate membranes supplied commercially for chemotaxis are coated with PVP to enhance their wettability. In this study, it was found that a substantial and variable number of migrated PMNs fell off the PVP-coated membranes. This made accurate quantification of chemotaxis difficult. When uncoated polycarbonate membranes were used and PMNs were suspended in protein-free medium, migrated neutrophils did not drop off filters after chemotaxis.

The new assay was adapted for use in the recently described 48-well

micro chemotaxis chamber (Falk et al., 1980). This has the advantage of ease of assembly and conservation of reagents (20,000 or fewer PMNs and 25 μl of chemotaxin per assay well). All neutrophils capable of migrating did so within 10—20 min. Individual neutrophils have been reported to migrate a distance of 10 μm within 1 min (Gamow and Barnes, 1974); therefore, the rate limiting step under the conditions of our assay might be settling of the cells onto the membrane surface.

Use of a 10 μm thick membrane lends itself to a determination of the fraction of PMNs that respond to chemoattractant, since migrated cells are in one optical plane on the bottom filter surface. The number of migrated cells, readily counted with an image analyzer, can then be compared with the input number. The fraction migrating is not 100%, and it may be of interest to determine whether this fraction is altered in disease. Because of the small number of PMNs required, the assay may be particularly useful in evaluating neutrophil chemotaxis of pediatric patients or others in whom the blood sample volume is a limiting factor.

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