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Specificity and Reversibility of Chemotactic Deactivation of Human Monocytes

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The chemotactic deactivation of human monocytes was studied to provide insight into the mechanism of chemotaxis. Deactivation was dependent on the dose of chemoattractant and time of incubation. A concentration in the cell suspension of 10^{-8} M *N*-formylmethionylleucyl phenylalanine (FMLP) for 45 min at 37°C led to 60% suppression of the subsequent specific chemotactic response. Higher concentrations of FMLP led to almost 100% specific suppression. Deactivation was specific under all conditions used. The response to a nonrelated chemoattractant, human serum-derived C5a, was unaffected by incubation in FMLP. Deactivation was also transient. If cells were deactivated at 37°C with FMLP, they recovered within 6 h at 37°C from this deactivation. Both phenomena, deactivation and recovery from deactivation, were temperature dependent. Monocytes could not be deactivated at 0°C, and they did not recover from deactivation when kept at 0°C. Thus, specific deactivation appears to require cellular metabolism, involving loss of receptors or blocking of a step between receptor occupancy and response.

Chemotactic deactivation refers to attractant-induced loss of migratory function, which is tested by reexposure of cells to chemotactic factor (19). Deactivation has been described for rabbit peritoneal polymorphonuclear leukocytes (20), human peripheral blood neutrophils (4, 9, 11, 12-14), and eosinophils (21). Human peripheral blood monocytes are also subject to deactivation (6, 8). Deactivation of human peripheral neutrophils has been dissociated into two different components. Low concentrations of chemotactic factor lead to inhibition of chemotactic response that is specific for the attractant, whereas high concentrations result in inhibition of the migratory system in a nonspecific manner. Several possible mechanisms have been proposed to explain these phenomena. Loss of an esterase activity (20) or damage to the cell by production of oxidative intermediates (13) or release of lysosomal enzymes (2) has been suggested for the mechanism of deactivation. Recently, specific deactivation of human peripheral blood neutrophils has been shown to correlate with loss of specific cell surface receptors for the attractant (16, 17). In this report, we show the dynamics of deactivation of human peripheral blood monocytes. Only specific deactivation was observed even when high concentrations of attractant or relatively long incubation times were used. We also show that, in contrast to human neutrophils, human monocytes are able to recover from deactivation.

MATERIALS AND METHODS

Cell preparation. Blood was drawn from healthy donors and heparinized. Mononuclear cells were isolated by the method of Boyum (3). Blood was diluted with an equal volume of Dulbecco phosphate-buffered saline without calcium or magnesium, and 35 ml of diluted blood was underlaid with 15 ml of Ficoll-Paque (Pharmacia Fine Chemicals, Inc., Piscataway, NJ.). After centrifugation at 400 × *g* for 40 min at 20°C, the interface cell layer was removed and washed twice with Gey balanced salt solution containing 2% bovine serum albumin (Cohn fraction V) (Gey-BSA; National Institutes of Health Media Unit, Bethesda, Md.). The cell suspension contained 15 to 35% monocytes, 65 to 85% lymphocytes, and less than 1% granulocytes. Viability was better than 99%, as measured by trypan blue dye exclusion. Total and differential counts were made for the final washed preparations. Total leukocyte recovery was 1×10^6 to 3×10^6 cells per ml of whole blood. Differential counts were made after staining cell suspensions with euchrysin 3RX (Roboz Surgical Instruments Co., Washington, D.C.) and observing them with a fluorescence microscope. These numbers were used to standardize the cell number added per well.

Chemotactic factors. *N*-Formylmethionylleucyl phenylalanine (FMLP) was purchased from Sigma Chemical Co., St. Louis, Mo. Stock solutions (10^{-3} M) were prepared in either ethanol or dimethyl sulfoxide and stored at −20°C. Human serum-derived complement component C5a was prepared by a modified procedure of Fernandez and Hügli (7) as described previously (6). Briefly, fresh serum was incubated with zymosan (Mann Research Laboratory) in the presence of ε-aminocaproic acid (Sigma Chemical Co.). C5a was

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then purified by consecutive chromatography on CM Sepharose Cl 6B (Pharmacia Fine Chemicals Inc.) and Sephadex G-100. The final preparation was stored as a solution in phosphate-buffered saline at −20°C.

Deactivation procedure. Cell preparations were diluted to concentrations of 1×10^5 to 2×10^5 monocytes per ml of Gey-BSA in 50-ml polypropylene tubes (no. 25335; Corning Glass Works, Corning, N.Y.). FMLP stock solution was diluted in Gey-BSA so that it could be diluted 1:100 into the cell suspensions. Standard incubation time for deactivation was 45 min unless indicated otherwise. After incubation, the cells were washed twice in medium at room temperature, and total and differential counts were made for the final suspensions.

For recovery experiments, the protocol was slightly changed. Cells to be tested for recovery at 0°C were put in ice immediately after deactivation, washed with cold medium, and centrifuged in the cold. Cells for testing at 37°C were washed and centrifuged at room temperature and then incubated at 37°C.

For time courses of deactivation and recovery from deactivation, cells were kept at the concentrations used for deactivation and tubes were frequently shaken. At the indicated times, samples were removed from the tubes and cells were centrifuged at $200 \times g$. Pellets were then suspended in Gey-BSA in 1/10 of the original sample volume. This suspension usually contained 1×10^6 to 2×10^6 monocytes per ml and was used for the chemotaxis assay.

Control cell suspensions were subjected to the identical protocol, but no FMLP was added for deactivation.

Chemotaxis assay. Chemotaxis was assayed in a multiwell chamber as described previously (5). Bottom wells were filled with 25 μl of attractant solutions in Gey-BSA. A Nuclepore filter sheet (Neuroprobe Inc., Bethesda, Md.; Polyvinylpyrrolidone coated, 10 μm thick, 5-μm hole size) was placed over the wells. Gasket and top plate were assembled, and 50 μl of cell suspension containing 50,000 to 100,000 monocytes, depending on the experiment, was added to each top well. Chambers were incubated in moist air containing 5% carbon dioxide for 90 min. After incubation, chambers were disassembled and filters were removed. Cells remaining on the top side were wiped off; filters were immersed in methanol for 15 s and then stained in Diff-Quick (Harleco, Gibbstown, N.J.). The number of cells per millimeter squared was counted with an image analyzer (between 1,500 and 3,000). With optimal concentrations of FMLP or C5a, 20 to 40% of the monocytes migrated toward the attractant, depending on the donor (6). These numbers were used to calculate percentage of control migration. Assay points were performed in triplicate, and the standard error of the mean was not greater than 15%.

RESULTS

Dependence of degree of chemotactic deactivation on dose of chemotactic factor. Monocytes were incubated at 37°C in various concentrations of FMLP to determine the range over which deactivation occurred. The cells were then washed, checked by trypan blue exclusion for viability (better than 99%), and tested for chemotaxis to FMLP and C5a. In all experiments, C5a served two purposes: as an indicator of cell viability and unimpaired migratory mechanism and, more important, as a discriminator between specific and nonspecific deactivation. The extent of deactivation, as measured by responses to optimal concentrations of FMLP and C5a, depended upon the concentration of FMLP used to deactivate (Fig. 1). An FMLP concentration of 10^{-8} M resulted in about 60% deactivation. Deactivation was complete at a concentration of 10^{-7} M. The response to C5a remained unchanged, except possibly at 10^{-5} M, the highest concentration of FMLP used. This indicates that deactivation was specific and that cell damage, at least as measured by chemotactic response, did not occur.

Deactivation did not lead to a shift in concentration of peptide needed for maximal chemotactic response. Dose responses for treated cells still capable of responding to the deactivating attractant were measured from 10^{-9} to 10^{-6} M, and the location of the response peak was the same as for the control.

The cell concentration used in deactivation experiments was between 1×10^5 and 2×10^5 monocytes per ml. Deactivation in suspensions with higher monocyte densities was unsuccessful; this was presumably due to loss of chemotactic factor, since supernatants of these suspensions showed decreased chemotactic activity.

Time course of deactivation. Monocytes were incubated with 10^{-7} M FMLP or in medium alone at 37°C. At intervals indicated in Fig. 2,

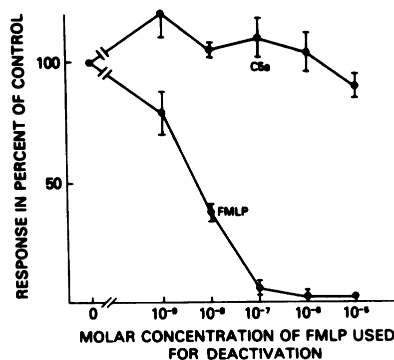

FIG. 1. *Dose-dependent specific deactivation of human monocytes. Mononuclear cells at a density of 2×10^5 monocytes per ml were incubated in FMLP solutions or medium at 37°C for 45 min. After two washes in Gey-BSA, monocyte chemotactic response to 10^{-8} M FMLP and a 1:800 dilution of C5a stock solution was measured. Error bars represent standard errors of the mean for triplicate assay wells.*

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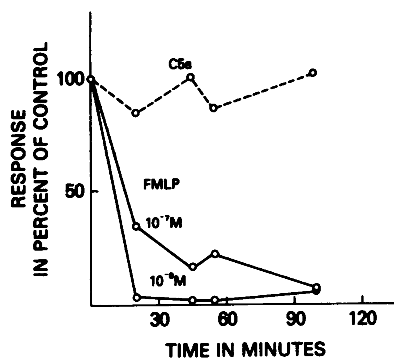

FIG. 2. *Time dependence of deactivation. Monocytes were incubated at 37°C in either 10^{-7} M FMLP or medium. At various times samples were removed, and cells were washed and assayed for chemotaxis to 10^{-7} and 10^{-8} M FMLP and to a 1:500 dilution of C5a. The data are presented as a percentage of the response of cells incubated in medium alone for the indicated times.*

equal portions were removed, washed, and assayed for their chemotactic response to C5a and FMLP at two different concentrations in the optimal range of the dose-response curve. Deactivation occurred very rapidly. After 20 min, deactivation was complete when the response to 10^{-8} M FMLP was tested and was 65% when the higher concentration was used. After 100 min, no response was detected at either concentration. The response to C5a remained constant throughout the experiment.

Temperature dependence of deactivation. To establish whether deactivation is simply blocking of receptors at the cell surface or an energy-requiring process, deactivation at 37 and 0°C was compared. Cells were incubated with 10^{-7} M FMLP or with medium as a control. Equal portions were removed at various times, washed, and tested for chemotactic response to C5a and three different concentrations of FMLP. One other set of cells was deactivated at 37°C with 10^{-7} M FMLP to show deactivation under normal conditions. The chemotactic response of cells deactivated at 37°C for 50 min was only 10% of the control (Fig. 3). No deactivation was detected in cells incubated at 0°C. The responses to C5a and optimal concentrations of FMLP were unchanged after 3.5 h. Only at a suboptimal concentration of FMLP (10^{-9} M) could some deactivation be observed. This marginal effect could be explained by the amount of FMLP bound at 0°C which might have caused partial deactivation when the cells were warmed to 37°C for the chemotaxis assay. These experiments show that deactivation requires a metabolizing cell; occupation of receptors at the surface, which occurs at 0°C (16, 17), is insufficient for deactivation.

Recovery of monocytes from deactivation. If deactivation is induced by receptor depletion, deactivated cells should be able to recover with time at 37°C. Monocytes were deactivated at 37°C by incubation in 10^{-7} M FMLP for 1 h. Control cells without FMLP were identically treated. The cells were then washed, and each tube was divided into two equal portions. One set was kept at 37°C in an incubator, the other was kept on ice, and at various times cells were tested for chemotaxis. Figure 4B shows the results with cells incubated at 37°C after deactivation. Deactivated monocytes gradually recovered from deactivation and reached control levels between 5 and 7 h. Recovery of the cells kept at 0°C reached 50% of the controls. Interpretation of this result is complicated by the fact that recovery can proceed during the assay in the chemotaxis well at 37°C. Thus, the 50% recovery of these cells represents the sum of possible recovery at 0°C and at 37°C during 90 min of incubation for the assay. Cell concentration during recovery did not affect the result. We cannot say with absolute certainty that there was no recovery at 0°C, but it must have been considerably slower than at 37°C. The control response to C5a shows again that monocyte chemotaxis function was unimpaired and that the events involving deactivation and recovery were specific for the attractant used for deactivation.

DISCUSSION

We report in this paper on the dynamics of chemotactic deactivation of human peripheral

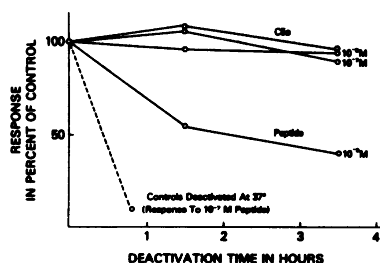

FIG. 3. *Deactivation of human monocytes at 0°C. Cells were incubated in either 10^{-7} M FMLP or medium at 0°C. Another set of cells was incubated at 37°C in either 10^{-7} M FMLP or medium. Deactivation at 37°C was terminated after 45 min; cells were washed twice and assayed for chemotaxis with 10^{-7} M FMLP as attractant. Cells deactivated at 0°C were washed twice at the indicated times, and the chemotactic response to FMLP (10^{-7}, 10^{-8}, and 10^{-9} M) and to a 1:500 dilution of C5a was measured. The control for each data point is the response of cells incubated in medium for the indicated deactivation time.*

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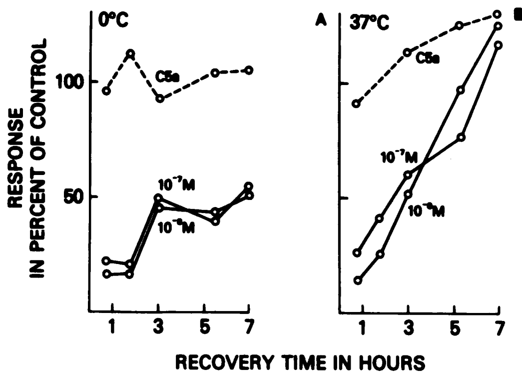

FIG. 4. *Recovery of human monocytes from deactivation. Cells were incubated at 37°C with 10^{-7} M FMLP or medium. After incubation, cells were washed twice to remove FMLP and divided into two portions each. One set of cells, incubated in FMLP or medium, was kept at 0°C (A), and one was kept at 37°C (B). At various times monocytes were tested for chemotactic response to FMLP (10^{-7} and 10^{-8} M) and to a 1:500 dilution of C5a. The figure combines two experiments. One experiment, in which the cell density during recovery was 2 × 10^6/ml, represents times of 20 min and 5 h 10 min. The remaining time points are for the other experiment, in which cell density was 2 × 10^5/ml.*

blood monocytes with FMLP as chemoattractant. Deactivation, or loss of chemotactic responsiveness after prior exposure to chemoattractants, did not occur at 0°C even after relatively long incubation periods. It proceeded very rapidly at 37°C, with almost total deactivation after 20 to 30 min of incubation. Deactivation and chemotaxis were also evident at room temperature (data not presented). The degree of deactivation was dependent upon the dose of attractant used, with a peptide concentration of 10^{-8} M leading to about 60% deactivation. Not only an optimal concentration of peptide but also a minimal absolute amount per cell was necessary. With monocyte concentrations of about 2 × 10^6 per ml, deactivation under the standard conditions (10^{-7} M FMLP) did not occur (data not shown). A likely explanation for this phenomenon is digestion of the peptide on or in the monocyte, since supernatants of these deactivations showed decreased chemotactic activity, indicating loss of chemotactic factor. Although release of FMLP split products has not yet been reported for monocytes, it has been shown for other cells (1, 16).

The deactivation of monocytes was always specific. In all experiments, we used the response to an unrelated attractant, C5a, as a control for specificity and functional capacity of the cell. Even at very long incubation times or at concentrations of attractant that were 100-fold higher than the concentration required for 90% deactivation, nonspecific deactivation was not observed. We have shown in a previous report (6) that this is also true for other attractants used for deactivation. This is in sharp contrast to the behavior of polymorphonuclear cells from rabbits and humans (12–14). Polymorphonuclear cell deactivation has been reported to have a specific and a nonspecific component, depending on the concentration of chemotatic factor used for deactivation. Only 40 to 50% deactivation could be achieved, and specific and nonspecific components could not be clearly separated. The nonspecific event leading to decreased cell motility was ascribed to several factors, such as (i) depletion of an enzyme or proenzyme pool or (ii) damage of the cell caused by oxidative intermediates. These mechanisms do not seem to operate in monocytes; if they do, the capacity of the cell for movement is not affected. Production of oxidative intermediates in monocytes has been shown to be poor compared with neutrophils (10, 15), and measured oxygen uptake by monocytes upon stimulation with FMLP is also very small (data not shown). Deactivation of monocytes for a specific attractant with retention of responsiveness to other attractants suggests that deactivation is at the level of the attractant receptor or at some point in the path leading to directional movement before the common pathway activated by all attractants. Since deactivation did not occur at 0°C, but required a metabolizing cell, binding to receptors by attractant appears to be insufficient for deactivation. Binding studies with labeled FMLP showed that the peptide was specifically bound at 0°C. The amount bound within 30 min of incubation was 70% of the amount bound at 22°C (W. Falk, L. Harvath, and E. J. Leonard, manuscript in preparation).

It has been reported for neutrophils and human monocytes (17; J. J. Muscato, J. E. Niedel, and J. B. Weinberg, Blood 54:89a, 1979) that incubation with chemotactic factor is followed by aggregation and internalization of the receptor-attractant complex. This is an energy-dependent phenomenon and is inhibited by low temperatures. This finding provides a plausible mechanism for specific deactivation in that it leads to depletion of the cells of receptors for that specific attractant. Into that scheme fits our observation that monocytes recovered from specific deactivation within about 6 h. The recovery was inhibited by low temperature. This time period correlates with the time monocytes need to recover from depletion of immunoglobulin G receptors (18), which has been shown to be approximately 6 h also. It does not mean that there must be a link between immunoglobulin G

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receptor and FMLP receptor, but this time span seems to be sufficient to rebuild the surface of the cell and elaborate new receptors. Experiments are in progress to correlate deactivation and chemotaxis with disappearance and reappearance of receptors on the surface.

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