# Insulin Degradation by Mononuclear Cells

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**SUMMARY**

**Mononuclear cells from peripheral blood possess insulin receptors that are altered in number or binding affinity in certain metabolic diseases as obesity. The monocyte, and not the lymphocyte, is the cell with the capacity to specifically bind insulin. Furthermore, this binding appears to mirror the receptor status on such insulin target tissues as liver, muscle, and fat. Since liver, muscle, and fat also degrade insulin, mononuclear cells from the blood of normal volunteers were examined for insulin-degrading activity. Intact cells were incubated with $^{125}$I-insulin and the amount of degraded insulin was measured by the trichloroacetic acid–precipitation technique. Insulin-degrading activity increased when the number of cells and the time of incubation were increased. Total insulin binding behaved in a similar fashion. Very little degradation was seen at 4° or 15°. The $K_m$ for insulin-degrading activity was 7.03 × $10^{-8}$ M. Homogenized mononuclear cells degraded two to five times more insulin than did intact cells and also demonstrated cell concentration, time, and temperature dependence for degradation. The $K_m$ of degradation for homogenized mononuclear cells was 2.2 × $10^{-8}$ M. Subcellular fractionation revealed significant degrading activity in the 100,000 × *g* supernatant, but little activity in the 100,000 × *g* pellet. A purified lymphocyte preparation did not bind insulin and contained little insulin-degrading activity.**

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Insulin-sensitive tissues, such as muscle,[1] liver,[2] fat,[3] and kidney,[4] have been shown not only to possess membrane receptors that specifically bind insulin but also to contain systems that degrade insulin. Studies have demonstrated insulin-degrading activity in isolated hepatocytes,[2,5] adipocytes,[3] in muscle fibers and homogenates,[1] in kidney and liver plasma membranes,[6,7] and in other subcellular fractions.[4,8] Kahn and Baird have suggested that, following the initial binding of insulin to a specific membrane receptor, the insulin was both altered and compartmentalized.[4] This parallels work by Ascoli and Puett, who proposed that human choriogonadotrophin binding to a specific membrane receptor is followed by an internalization of this receptor-ligand complex.[9] The work of Schlessinger and co-workers demonstrated an internalization of fluorescently labeled insulin following the incubation of insulin and fibroblasts.[10]

Mononuclear cells from peripheral blood possess insulin receptors[11,12] that are altered in number or binding affinity in certain metabolic diseases as obesity.[13,14] The monocyte and not the lymphocyte is the cell with the capacity to specifically bind insulin.[15] Furthermore, this binding appears to mirror receptor status in such insulin-sensitive tissues as liver and fat.[12] Since liver, muscle, and fat all degrade insulin, we have examined human mononuclear cells for insulin-degrading activity. Herein, we demonstrate that both intact and homogenized mononuclear cells and their subcellular fractions actively degrade insulin. The insulin-degrading mechanism is characterized and the effects of time and temperature examined.

## MATERIALS AND METHODS

**Materials.** Bovine serum albumin (BSA) was obtained from Miles Laboratories and HN-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) from Calbiochem. Pork crystalline zinc insulin, from Dr. R. Chance of Lilly Research Laboratories, was prepared as a stock solution and diluted in 0.1M HEPES-1% BSA buffer, pH 7.4, to the desired insulin concentrations. $^{125}$I-insulin was obtained from Immuno Nuclear Corporation at an activity of 150–200 $\mu$Ci/$\mu$g and further purified by chromatography on DEAE cellulose column and the appropriate peaks were diluted in HEPES-BSA buffer.

**Isolation of mononuclear cells.** Blood from normal, fasted human volunteers was placed in heparinized containers and diluted 1.1 with phosphate-buffered saline (PBS), pH

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7.4. The mononuclear cells were isolated by the method of Boyum.[16] Ficoll-Hypaque was added to the PBS-diluted blood and centrifuged at 500 × *g* for 40 min at room temperature in an International Equipment centrifuge. The mononuclear cell layer was removed and suspended in a 0.1M HEPES-1% BSA buffer, pH 7.4. The number of mononuclear cells was quantitated with a hemacytometer. Cell viability was assessed by trypan blue exclusion and found to be consistently greater than 95%. As estimated by a nonspecific esterase stain,[17] monocytes comprised from 15 to 20% of the mononuclear preparation.

**Insulin degradation by intact mononuclear cells.** A suspension of 25 × $10^6$ mononuclear cells was added to $^{125}$I-insulin at a concentration of 5 × $10^{-11}$ M (0.3 ng/ml) and diluted to a total volume of 500 μl by the addition of HEPES-BSA buffer, pH 8.0. This mixture was incubated for 30 min at pH 8.0 and 37°C. Experiments were performed that varied in the number of mononuclear cells, the length of incubation, and the temperature of incubation. To one incubation medium, no $^{125}$I-insulin was added. This medium was incubated under the customary conditions and then placed in a Beckman microfuge and spun at 13,000 × *g* for 5 min. The resulting cell pellet was discarded, and the supernatant was combined with $^{125}$I-insulin and incubated for 30 min at pH 8.0 and 37°C. An aliquot of this supernatant was then analyzed for degrading activity as outlined below. After incubation with labeled insulin, 200-μl aliquots were removed from the remaining incubation mixtures and placed in plastic microfuge tubes containing 150 μl of HEPES-BSA buffer, pH 8.0, at 4°C. A cell pellet was obtained by centrifuging the mixture at 13,000 × *g* for 5 min at 4°C in a Beckman microfuge. The resulting supernatant was combined with trichloroacetic acid (TCA) at a final concentration of 5% TCA. Binding and degradation were determined as described previously.[18,19] Nonspecific binding averaged about 20% of the total binding, in agreement with previous studies.[13,20]

**Preparation of homogenized mononuclear cells and subcellular fractions.** Mononuclear cell homogenates were prepared from a suspension of cells obtained by the Ficoll-Hypaque method outlined above. After suspension in a 0.35M sucrose solution, the cells were homogenized in a hand-powered, glass on glass, Pyrex homogenizer and then partitioned into an incubation mixture at a concentration of 5 × $10^6$ cells. The mixture was brought to a final volume of 500 μl with the addition of HEPES-BSA buffer, pH 7.4, and incubated for 30 min at pH 7.4 and 37°C. Aliquots of 200 μl were removed immediately after incubation to a solution containing a final concentration of 5% trichloroacetic acid.[18,19]

Subcellular fractions were obtained as follows. Mononuclear cells were diluted in 0.1 mM $NaHCO_3$ and homogenized. This homogenate was centrifuged at 500 × *g* for 30 min. The supernatant produced was recentrifuged at 30,000 × *g* for 30 min at 4°C in a Beckman model L5-65 ultracentrifuge. The resulting supernatant was centrifuged at 100,000 × *g* for 30 min at 4°C. The three pellets and the 100,000 × *g* supernatant were incubated with labeled insulin at pH 7.4 and 37°C. Aliquots were analyzed for degraded insulin by the TCA method.

**$K_m$ determinations.** The intact mononuclear cell preparations, the mononuclear homogenates, and the subcellular fractions were incubated under identical conditions with labeled insulin and varying amounts of unlabeled insulin. The $K_m$ of the insulin-degrading mechanism was calculated by the customary Lineweaver-Burke plot and linear regression analysis.

**Lymphocyte and monocyte preparations.** Lymphocyte and monocyte preparations were obtained from Dr. Arnold Postlethwaite (Veterans Administration Medical Center, Memphis, Tennessee). Mononuclear cells were prepared from whole blood by the method of Boyum[16] and separated according to cell size by countercurrent centrifugation. These were 95% pure lymphocytes and 60% pure monocytes, by staining with nonspecific esterase.

**Assay of binding and degradation by lymphocytes and monocytes.** Binding of $^{125}$I-insulin was determined as reported previously.[13] A lymphocyte preparation with 20 × $10^6$ cells was incubated with $10^{-11}$ M $^{125}$I-insulin (0.3 ng/ml) for 90 min at pH 8.0 and 22°C. After incubation, a 200-μl aliquot was spun in a Beckman microfuge. The supernatant and the pellet were quantitated for radioactivity and the net percent insulin bound to the lymphocytes was calculated from the radioactivity in the cell pellet over the total radioactivity in the cell pellet and the supernatant.

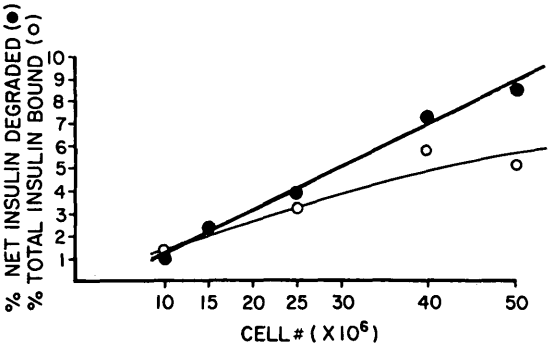
A lymphocyte preparation and a monocyte suspension were homogenized in a 0.35M sucrose solution as outlined above. The lymphocyte homogenate and the monocyte homogenate were incubated for 60 min at pH 7.4 and 37°C. The percent insulin degraded was derived as previously mentioned.

Degradation by a 95% pure intact lymphocyte preparation and a 60% pure intact monocyte preparation (the remaining cells being large lymphocytes) was measured as outlined above.

## RESULTS

**Insulin degradation by intact mononuclear cells.** Figure 1 is a diagram of the relation between the amount of insulin degraded and the number of mononuclear cells placed in the incubation medium. Total binding of insulin also exhibited a similar response to increasing cell concentration. It should be noted that the total binding is not corrected for nonspecific binding. As a control, mononuclear cells were incubated without labeled insulin under identical circumstances and then removed by centrifugation. The resulting supernatant did not degrade insulin.

**FIGURE 1. Insulin degradation and binding at varying cell concentrations (expressed as by $10^5$ cells in each 500 μl). Mononuclear cells were incubated with $^{125}$I-insulin for 30 min at pH 8.0 and 37°C in 0.1 M HEPES-1% BSA buffer. The percent insulin degraded was measured by TCA precipitation. The incubation mixture did not degrade insulin after removal of the mononuclear cells.**



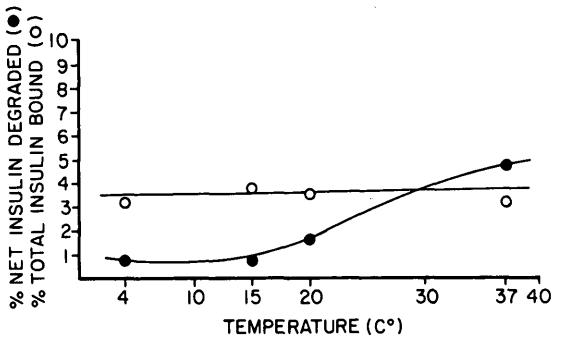



**FIGURE 2. Insulin degradation and binding at varying incubation temperatures (°C). A volume of 500 μl, containing 25 × $10^6$ mononuclear cells and $^{125}$I-insulin, was incubated for 30 min at pH 8.0 and 4°C, 15°C, 20°C, and 37°C. The percent insulin degraded was measured by TCA precipitation. The incubation mixture did not degrade insulin after removal of the mononuclear cells.**

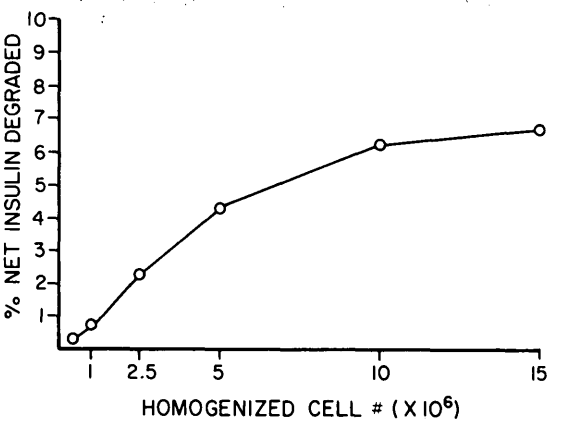



**FIGURE 4. Insulin degradation by homogenized mononuclear cells at varying cell concentrations (expressed as by $10^6$ homogenized cells in each 500 μl). Homogenized mononuclear cells were incubated with $^{125}$I-insulin for 30 min as pH 7.4 and 37°C in 0.1M HEPES-BSA buffer. The percent insulin degraded was measured by TCA precipitation.**

When insulin degradation was plotted against the temperature of incubation, the resultant curve is shown in Figure 2. Total insulin binding changed little over the temperature range examined. After removal of the mononuclear cells, the incubation mixture did not degrade insulin.

As shown in Figure 3, increasing the time of incubation produced an appropriate increase in the amount of insulin degraded. Unlike the studies shown in Figures 1 and 2, however, the incubation medium possessed insulin-degrading activity after removal of the cells. The amount of degrading activity in the cell-free medium depended on the length of incubation and represents the major percentage of degrading activity measured. Incubation of mononuclear cells and labeled insulin for 30 min produced an average of 5.46% of the insulin degraded, while the media following removal of the cells degraded 4.27% of the insulin at the end of a 30-min incubation. This activity in the media represents either continuous leakage from the cells or degrading activity that is only loosely attached to the cell surface. Various adjustments in the experimental protocol, as balanced salt buffers and preincubation of the cells before adding the labeled insulin, failed to prevent this extracellular degradation. Total insulin binding increased with longer periods of incubation and appeared to reach a maximum at 60 min.

**Insulin degradation by homogenized mononuclear cells.** Homogenized mononuclear cells degraded approximately fivefold more insulin than did intact cells (Table 1). This degradation is related both to the number of cells in each homogenate and the length of incubation, as shown in Figures 4 and 5, respectively. Figure 6 indicates the relationship between degradation and the temperature of incubation by homogenized mononuclear cells.

**$K_m$ determination.** Using a Lineweaver-Burke plot and linear regression analysis, the $K_m$ for the insulin-degrading mechanism was determined. For intact cells, the $K_m$ was 7.03 ×$10^{-8}$ M; for homogenized mononuclear cells, the $K_m$ was 2.2 ×$10^{-8}$. The incubation medium possessed insulin-degrading activity after the removal of cells. Table 2 contains a comparison of the kinetic constants of degradation for intact and homogenized cells.

**FIGURE 3. Insulin degradation and binding at increasing lengths of incubation (minutes). A volume of 500 μl, containing 25 × $10^6$ mononuclear cells and $^{125}$I-insulin, was incubated at pH 8.0 and 37°C for increasing periods of time. The percent insulin degraded was measured by TCA precipitation. The incubation mixture after the removal of the mononuclear cells degraded a significant amount of insulin.**

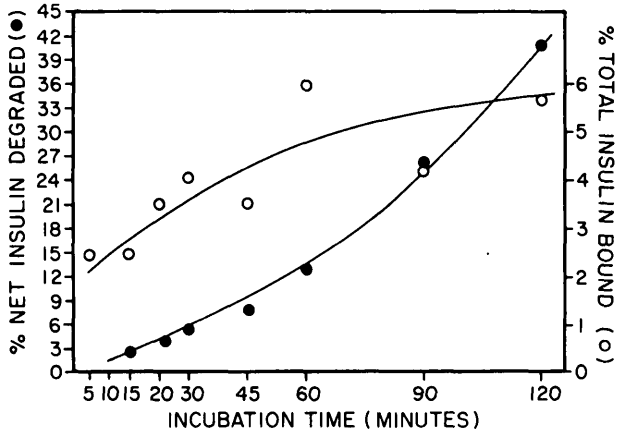



**FIGURE 5. Insulin degradation by homogenized mononuclear cells at increasing lengths of incubation (minutes) shown along the x axis. A volume of 500 μl, containing 5 × $10^6$ homogenized mononuclear cells and labeled insulin, was incubated at pH 7.4 and 37°C for increasing periods of time. The percent insulin degraded was measured by TCA precipitation.**

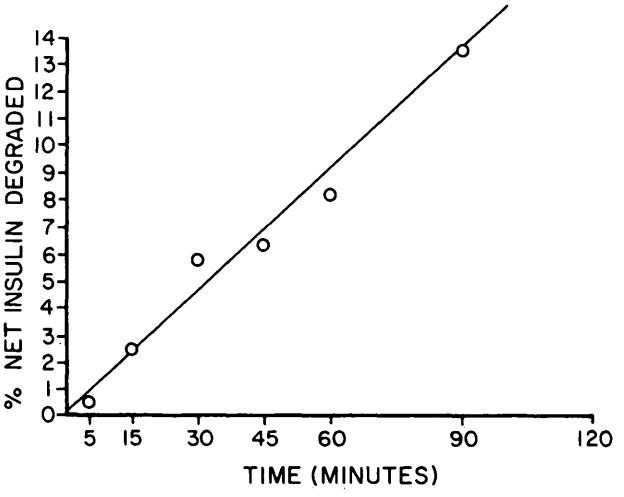


TABLE 1
Insulin degradation by intact and homogenized mononuclear cells

| Intact cells | Insulin binding | Insulin degraded (moles) | Total degrading activity |
|---|---|---|---|
| Total mononuclear cells ($25 \times 10^6$ cells) | Yes | $5.46 \times 10^{-13}$ | 100% |
| Intact lymphocytes ($20-21.25 \times 10^6$ cells*) | None detected | None detected | 0% |
| Intact monocytes ($3.75-4.0 \times 10^6$ cells*) | Not assessed | $2.5-3.3 \times 10^{-13}$ | 46–61% |
| Total mononuclear cells ($5 \times 10^6$ cells) | | $5.73 \times 10^{-13}$ | 100% |
| Homogenized lymphocytes ($4.0-4.25 \times 10^6$ cells*) | | $1.78-1.89 \times 10^{-13}$ | 31–33% |
| Homogenized monocytes ($0.75-1.0 \times 10^6$ cells*) | | $1.34-1.79 \times 10^{-13}$ | 23–31% |

* This represents the approximate number of these cells in the mononuclear cell preparation. All incubations were at 37° for 30 min.

**Insulin degradation by mononuclear subcellular fractions.** Table 3 illustrates the amount of insulin-degrading activity present in a $500 \times g$, a $30,000 \times g$, and a $100,000 \times g$ pellet and in the $100,000 \times g$ supernatant. Most of the activity resided within the cytosol fraction. Only small amounts of activity were found in the $500 \times g$, the $30,000 \times g$, and the $100,000 \times g$ pellet. The cells were homogenized in 0.1 mM $NaHCO_3$, and this released considerably more degrading activity than did homogenizing the cells in a 0.35 M sucrose solution.

The $K_m$ constant of the cytosol-associated insulin-degrading system was $1.97 \times 10^{-7}$ M (R = 0.985), as determined by a Lineweaver-Burke plot and linear regression analysis.

**Insulin binding and degradation by lymphocytes and monocytes.** A 95% pure lymphocyte preparation exhibited no ability to specifically bind insulin.

An intact lymphocyte preparation of 95% purity possessed no significant insulin-degrading activity; an intact monocyte preparation of 60% purity, with no significant degrading activity in the media, degraded $1.96 \times 10^{-13}$ mol of insulin in 30 min ($5 \times 10^6$ cells). Homogenized lymphocytes of 95% purity degraded $2.31 \times 10^{-13}$ mol of insulin in 30 min ($5 \times 10^6$ cells), while a homogenized monocyte preparation of 60% purity degraded $5.4 \times 10^{-13}$ mol of insulin in 30 min ($5 \times 10^6$ cells). Large lymphocytes constituted the remaining percentage of cells in the monocyte preparation (Table 1).

## DISCUSSION

The relation of insulin binding by membrane receptors and insulin degradation is uncertain, but studies have suggested that receptor interaction is a necessary prerequisite for insulin degradation.[2,21] Gliemann and Sonne have concluded that degradation of receptor-bound insulin does occur.[22] Terris and Steiner have shown that inhibition of insulin binding by the addition of unlabeled insulin or by treatment with trypsin produces an equal degree of depression of insulin degradation.[2] Following the incubation of intact hepatocytes and iodinated insulin, the demonstration of an unalterable lag phase preceding the appearance of degradation products supports the concept of a mandatory transport of the bound insulin to a membrane-associated or intracellular site.[2] This is consistent with the recent work of Kahn and Baird, in which insulin was both altered and compartmentalized after being bound to adipocytes.[3] The form and location of the internalized insulin is unknown. Schlessinger et al. have visualized the sequence of insulin binding, receptor aggregation, and intracellular uptake of the insulin label, and this series of reactions depends on time, temperature, and energy.[10]

In this study, intact mononuclear cells were shown to actively degrade insulin in a time-, temperature-, and cell concentration–dependent manner. As shown in Figures 1 and 3, both total insulin bound and insulin degraded increased with length of incubation and enlargement of the cell concentration. Insulin degradation displayed a temperature-dependent relationship. However, total insulin binding (specific insulin bound plus insulin nonspecifically bound) appeared unrelated to the temperature of incubation. This is in agreement with previously reported insulin receptor activity in kidney and liver membranes[6] and mononuclear cells,[23] in which insulin binding was of comparable magnitude—regardless of incubation temperature—for incubations of less than 45 min. With greater lengths of incubation, differences in insulin binding with varying temperatures of incubation have been reported.[6,23] Maximum insulin-degradative activity occurred at 37°C, with only minor amounts of

**FIGURE 6. Insulin degradation by homogenized mononuclear cells at varying incubation temperatures (°C). A volume of 500 μl, containing $5 \times 10^6$ homogenized mononuclear cells and $^{125}$I-insulin, was incubated for 30 min at pH 7.4 and 4°C, 15°C, 20°C, and 37°C. The percent insulin degraded was measured by TCA precipitation.**

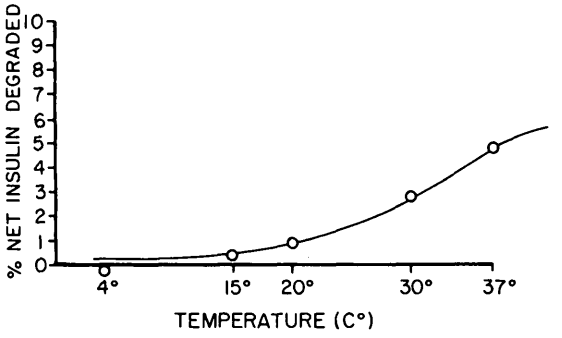



TABLE 2
Kinetic constants for insulin degradation by intact and homogenized mononuclear cells

| | $K_m$ ($\times 10^{-8}$ M) | $\times 10^{-10}$ mol $V_{max}$ ($5 \times 10^6$ cells, 30 min, 37°C) | R |
|---|---|---|---|
| Intact cells | 7.03 | 5.66 | 0.9809 |
| Homogenized cells | 2.2 | 11.93 | 0.9890 |

TABLE 3
Subcellular fractionation of insulin-degrading activity*

| | $< 10^{-9}$ mol of insulin degraded | % of total degrading activity |
|---|---|---|
| Complete mononuclear homogenate | 20.2 | 100 |
| 500 × *g* pellet | 0.96 | 4.8 |
| 30,000 × *g* pellet | 0.74 | 3.7 |
| 100,000 × *g* pellet | 0.56 | 2.8 |
| 100,000 × *g* supernatant | 15.6 | 77.2 |

* Activity for equivalent of $5 \times 10^6$ cells, at 37°C, for 30 min.

degraded products found with incubation temperatures of 4°C and 15°C, and an intermediate quantity of degradation at 22°C. Similar temperature relationships are seen in liver membranes,[6,7] kidney membranes,[6] isolated hepatocytes,[5,7] and fat cell membranes.[8]

After incubation, the intact mononuclear cells were removed by centrifugation and the remaining media were examined for degrading activity. During the studies on optimum cell concentration and temperature of incubation, the incubation media minus the mononuclear cells did not degrade insulin. However, the data obtained for varying lengths of incubation represent degradation by intact mononuclear cells and by degrading activity in the media. In twenty separate attempts, the incubation media's degradation accounted for the major portion of the degradation measured. We were unable to alter the experimental protocol to rid our experimental system of this cell-independent-degrading activity. Since samples of the cell-free suspension at various points in the isolation of the mononuclear cells contained degrading activity, it was felt that this media activity represents cell trauma and rupture by the experimental procedure. Gross cell viability, as assessed by trypan blue exclusion, was always greater than 95%, however.

Homogenized mononuclear cells degrade two- to fivefold more insulin than do intact cells. Hammond and Jarett have demonstrated a fourfold difference in degradative activity in homogenized and intact fat cells.[8] The mononuclear cell homogenates exhibited similar relationships to length and temperature of incubation as intact cells.

The $K_m$ for intact mononuclear cells, as determined by a Lineweaver-Burke plot and linear regression analysis, was $7.03 \times 10^{-8}$ M. Olefsky has reported a $K_m$ of $5 \times 10^{-7}$ M for insulin degradation by hepatocytes,[7] Le Cam et al. a value of $2.7 \times 10^{-7}$ M for hepatocytes,[5] and Duckworth a value of $2.2 \times 10^{-7}$ M for kidney membranes[6] and $2.2 \times 10^{-8}$ M for insulin protease isolated from muscle.[1]

As shown in Table 3, most of the insulin-degrading activity following subcellular fractionation resides in the 100,000 × *g* fraction. This is consistent with several previous subcellular fractionation studies,[4,5,24] all of which located the major portion of insulin-degrading activity in the cytosol fraction. The $K_m$ of degradation by the cytosol fraction is $1.97 \times 10^{-7}$ M and this is comparable to the $K_m$ for the total mononuclear cell homogenate and the $K_m$ determinations from other investigators.[1,5-7] However, there is almost a 10-fold difference in homogenate and cytosolic $K_m$. It is possible the homogenate contains materials that interact with the enzyme in some way to alter the kinetic properties of the enzyme. Other tissues, specifically liver and kidney, do contain activators and inhibitors of insulin protease. In general, caution must always be taken in interpreting kinetic values in crude preparations.

Our present study indicates that intact lymphocytes of 95% purity possess insignificant insulin-binding and -degrading capability. The inability of circulating lymphocytes to bind insulin is well known,[11,12,23,25] although PHA-stimulated lymphocytes do contain receptors.[26] In contrast, isolated monocytes of 60% purity (the remainder of the cells being large lymphocytes) degraded $1.96 \times 10^{-13}$ mol of insulin for each $5 \times 10^6$ cells in 30 min. The total mononuclear cell suspensions, composed of from 15 to 20% monocytes with the remainder being lymphocytes, degraded $5.46 \times 10^{-13}$ mol for each $25 \times 10^6$ cells in 30 min. Therefore, it appears that the monocyte accounts for the major degradative activity demonstrated in intact mononuclear cell suspensions (Table 1).

Mononuclear cells from peripheral blood degrade insulin in a time- and temperature-dependent fashion. The major insulin-degrading activity in intact mononuclear cell preparations resides in the monocyte, since intact lymphocytes exhibit little ability to bind or degrade insulin. Homogenized mononuclear cells, which degrade two- to fivefold more insulin than do intact cells and lymphocytes, are responsible for only slightly more of this degradation than are monocytes—despite their fivefold greater representation in the homogenate. Homogenized monocytes degrade approximately fourfold more insulin than a like number of homogenized lymphocytes. The cytosol is the site for the major portions of this activity. The location and the $K_m$ of insulin degradation for mononuclear cells closely resemble the characteristics of insulin protease and insulin degradation in such insulin-responsive tissues as liver, muscle, and fat. Mononuclear cell preparations may prove useful in the study of the insulin-degrading mechanism.

**ACKNOWLEDGMENTS**

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