Monocyte Localization of Elevated cAMP Phosphodiesterase Activity in Atopic Dermatitis

Colin A. Holden, B.Sc., M.R.C.P., Sai Chung Chan, M.S., and Jon M. Hanifin, M.D.
Department of Dermatology, The Oregon Health Sciences University, Portland, Oregon, U.S.A.

Patients with atopic dermatitis (AD) manifest a number of immune abnormalities which correlate with in vitro defects including lymphocyte transformation, chemotaxis, and cytotoxicity. Past studies have shown reduced leukocyte cyclic 3’,5’-adenosine monophosphate (cAMP) levels after exposure to adenylyl cyclase-active agonists, and we have demonstrated that this results from increased catabolism due to elevated cAMP-phosphodiesterase activity. These results were obtained in preparations containing mixtures of lymphocytes and monocytes. In order to determine more precisely the cellular site of the defect we have separated the leukocytes into lymphocyte- and monocyte-enriched preparations using either Percoll-gradient centrifugation or adherence isolation. Both techniques yielded over 93% pure lymphocytes, whereas the former yielded 64% monocytes compared with the latter method which generated 94% pure monocytes. Atopic monocytes, obtained by either technique, consistently showed elevated phosphodiesterase activity compared with those of the nonatopic monocytes. Such differences were not evident in lymphocyte preparations from normal and atopic subjects. In spite of the increased rate of cAMP degradation in atopic leukocytes, the resting cAMP levels do not differ from those of normal subjects. We questioned whether this is caused by increased cAMP synthesis and evaluated cellular adenylyl cyclase activity. We found no evidence in AD cells for an increased rate of adenylyl cyclase catalysis, either basal activity or after stimulation by forskolin. Therefore, the resting cAMP levels must have been compensated by other mechanisms. Impaired cyclic nucleotide metabolism in atopic monocytes may affect a number of immunologic and inflammatory reactions and could account for many of the clinical abnormalities in atopic diseases. J Invest Dermatol 87:372–376, 1986
in leukocyte homogenates from normal subjects and patients with AD.

**MATERIALS AND METHODS**

**Mononuclear Leukocyte Isolation** Heparinized venous blood was taken at 9:00 AM from 13 young adult patients with typical AD [1], ranging from mild to severe, and from 12 normal healthy young adult nonatopic control subjects. Patients were receiving no medications and had ingested no caffeine or other methyl xanthine-containing beverages for at least 14 h. The MNL were separated on Hypaque-Ficoll (HF) gradients (Ficoll-Paque, Pharmacia, Piscataway, New Jersey) by centrifugation of equal volumes of blood and normal saline at 450 g for 30 min. The cells were washed three times in Hanks’ calcium- and magnesium-free buffer (Hanks’ CMF, pH 7.6) at 400 g, 300 g, and 250 g to reduce platelet numbers and then were suspended in Hanks’ CMF at 20–30 × 10⁶ cells/ml.

**Percoll Separation** Continuous gradients were formed by combining 7 parts Percoll (Pharmacia) with 6 parts of a double-strength phosphate-buffered saline (PBS, pH 7.4). Fifteen-milliliter aliquots were centrifuged in 16 ml polycarbonate tubes (no. 257; Sorvall-Dupont Inst., Rockville, Maryland) at 25,000 g in an angle head rotor (SS-34) in a Sorvall RC-5B, to form the gradients.

Twenty to thirty million MNL were layered onto the gradients in 1 ml of Hanks’ CMF and spun at 1,000 g in a hanging bucket rotor. Two bands were produced: an upper, monocyte-rich (MR) fraction and a lower, lymphocyte-rich (LR) fraction as described by Gmelig-Meyling and Waldman [15].

The cells were washed twice with Hanks’ CMF and resuspended at 15–20 × 10⁶/ml in Hanks’ CMF for the adenylate cyclase assay or at 2 × 10⁶/ml in Gey’s balanced salt solution (GBSS, Gibco, Grand Island, New York) for the PDE assay.

**Adherence Separation** For adherence purification of the monocytes we processed heparinized blood in a modification of the methods of Pawlowski et al [16]. Whole blood was washed twice with equal volumes of calcium- and magnesium-deficient PBS (PD) at 4°C. The blood cells were then resuspended at original volume, in PBS containing 0.3 mM EDTA and layered onto 4 ml HF in 15-ml plastic tubes (no. 3026, Falcon, Oxnard, California) for separation of MNL. The MNL were washed and then resuspended in 1 ml of PD for counting. An aliquot was diluted with GBSS to a cell count of 1–2 × 10⁶ cells/ml for PDE assay. The remainder of the cells was diluted to 10⁶/ml in RPMI-1640 (Gibco) containing 15% human AB serum. Three milliliters of this suspension were incubated for 2 h in a 60-mm plastic dish (Corning no. 25010, Corning, New York) at 37°C in 5% CO₂/95% air to allow monocyte adherence.

The supernatant was aspirated and the dishes were rinsed 3 times with warm GBSS and gentle agitation to remove nonadherent cells. A coverslip, previously placed in the bottom of the dish, was removed for adherent cell characterization. The adherent cells were washed from the plastic by vigorous pipetting with PD. Complete removal and dissociation of monocytes were monitored using an inverted phase microscope. The adherent and nonadherent cell populations were washed and resuspended at 1–2 × 10⁶/ml GBSS for PDE assay.

**Cell Characterization** Cell viability from both separation methods, monitored by trypan blue exclusion, was always greater than 95%. The MNL, MR, LR, supernatant cells, and adherent cells were characterized by Giemsa and a naphthyl acetate esterase (ANAE) stains [17], and by latex bead uptake. Values were reported as the mean of the percentages obtained by the 3 methods.

**Cyclic AMP Phosphodiesterase Activity** Cells at 1–2 × 10⁶/ml in GBSS were passed through 3 freeze-thaw cycles and sonicated for 2 min at 4°C by a Braun-Sonics 2000 (B. Braun, Melsungen, F.R.G.) set at 50 W. The homogenate was assayed for PDE activity using a modification of the procedure of Thompson et al [18].

To 200 μl of the sample was added 200 μl of substrate containing 1 μM cAMP, 20,000 cpm [H]cAMP (New England Nuclear, Boston, Massachusetts) in 40 mM Tris-HCl buffer (pH 8.0) containing 3.75 mM β-mercaptoethanol and 50 mM MgCl₂.

After a 10-min incubation at 30°C the reaction was stopped by boiling for 45 s and snap-freezing in an ethanol/dry ice mixture. The mixtures were incubated subsequently with 100 μl of cobalt venom (1 mg/ml, Sigma Chemical Co., St. Louis, Missouri) for 10 min at 30°C and then mixed with 1 ml of AGI ×2 resin (200–400 mesh, BioRad, Richmond, California) for 30 min at 4°C.

The samples were counted in Ready-Solv (Beckman Instruments Corp., San Jose, California) and enzyme activity was expressed as pmol cAMP hydrolyzed per minute per 10⁶ cells.

**Adenylate Cyclase Assay** Cells 2 × 10⁷/ml in Hanks’ CMF (pH 7.4) were freeze-thawed 3 times and homogenized 40 strokes in a Dounce homogenizer. Nonstimulated and forskolin-stimulated AC activities were determined using a modification of Salomon’s method [19].

The final reaction volume of 50 μl contained 20 μl of MNL homogenate, 10 μl of forskolin (10⁻⁵ M, Sigma) or distilled water, 10 μl of 10⁻⁴ M PDE inhibitor R2020-1724 (provided by J. M. Kwon, Hoffman-LaRoche, Nutley, New Jersey), and 10 μl of assay cocktail, to give a final concentration of reagents as follows: creatine phosphate 3 mM, creatine phosphokinase 50 units/ml, adenine triphosphate (ATP) 0.5 mM, cAMP 0.05 mM, dihydrotestosteron 1 mM, bovine serum albumin 0.1 mg/ml, guanosine 5'-triphosphate (GTP) 0.01 mM, and magnesium acetate 5 mM (all from Sigma Chemical Co.), and [³²P]ATP 2–6 × 10⁶ cpm per assay (New England Nuclear) in Tris acetate buffer 25 mM (pH 7.6).

Duplicates were incubated for 1 h at 36°C and the reaction ended by mixing with 100 μl of stopping solution composed of 2% lauryl sulfate, 45 mM ATP, and 1.3 mM cAMP (Sigma). Assay blanks were prepared by adding cell homogenate after stopping solution and by omitting the homogenate completely. All tubes contained 50 μl of [H]cAMP to monitor cAMP recovery. Cyclic AMP was recovered by passing the 200-μl aliquots plus 1 ml deionized water through Dowex AGWX4 columns (200–400 mesh BioRad). After further flushing with 1 ml of water, the Dowex columns were placed over alumina columns and washed with 9 ml of deionized water. The alumina columns were then flushed with 6 ml of imidazole buffer (pH 7.3) and the 6-ml eluates were collected in vials containing 14 ml of scintillation fluid (Ready-Solv, Beckman Instruments, Inc., Fullerton, CA). The recovery of cAMP was 75–90% and the specific activity of AC was expressed as pmol cAMP produced per minute per 10⁶ cells.

**RESULTS**

**Mononuclear Leukocyte Subpopulations** (Table I) Hypaque-Ficoll separation yielded lymphocytes and monocytes of greater than 99% purity in yields consistently exceeding 80% of total cells.

**Table I.** Composition of Atopic and Normal Mononuclear Leukocyte (MNL) Subsets from Hypaque-Ficoll, Percoll-Separated Lymphocyte-rich (LR) and Monocyte-rich (MR) Cell Fractions and Adherence-Separated Monocyte and Lymphocyte Preparations

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<tr>
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<th>Atopic Dermatitis</th>
<th>Normal</th>
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<tr>
<td>MNL</td>
<td>70 ± 3.7% (L)</td>
<td>70 ± 2.5% (L)</td>
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<tr>
<td>MR</td>
<td>64 ± 4.5% (M)</td>
<td>60 ± 4.9% (M)</td>
</tr>
<tr>
<td>LR</td>
<td>94 ± 2% (L)</td>
<td>93 ± 2.6% (L)</td>
</tr>
<tr>
<td>Adherent</td>
<td>94 ± 5% (M)</td>
<td>93 ± 3.4% (M)</td>
</tr>
<tr>
<td>Nonadherent</td>
<td>91 ± 3.3% (L)</td>
<td>90 ± 4.6% (L)</td>
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*Mean % ± SEM of lymphocytes (L) or monocytes (M).
MNL and 95% of monocytes. We found no differences between the proportions of monocytes and lymphocytes in the MNL, or in Percoll-riched LR and MR preparations from AD patients and normal nonatopic subjects. The MNL preparations contained 30% monocytes and this was increased to 64% in the MR fractions. The LR fractions were composed of an average of 94% lymphocytes. Mean total cell recovery was 92.8% with 90.4% monocyte and 93.8% lymphocyte recovery from the Percoll gradients. There were no differences in recoveries of normal and AD cells.

The adherence method provided monocyte preparations of 94% and 93% purity in preparations from AD patients and normal subjects, respectively. Seventy to eighty percent of the MNL remained nonadherent and these supernatant cells consisted of 91% and 90% lymphocytes in AD and normal subsets, respectively. In addition, some preparations were examined for nonphagocytic cells bearing surface immunoglobulin. These revealed that the nonadherent cells contained approximately 10% B cells, compared with only 2% B cell contamination of adherent cells. No differences in percentages were seen between the AD (n = 2) and normal samples examined (n = 4).

In contrast, the MR fraction contained 11% B cells whereas the LR fraction contained 3% B cells. Again, no differences were apparent between the AD and normal preparations.

**Phosphodiesterase Activity**

**Percoll Separation:** The PDE activity varied, depending on the subpopulation examined (Table II). Normal and AD subjects showed no significant difference between MNL and MR preparations. The PDE activity in LR fractions was significantly less than that in MNL and MR populations (p < 0.005) but no significant difference existed between AD and normal LR cells. In contrast the AD preparations showed a significantly higher activity in both MNL fractions compared with normal (p < 0.005). It was observed that, in spite of a higher proportion of monocytes, the PDE activity of the Percoll-separated MR fraction was similar to that of the MNL. Thus, there appeared to be a Percoll-induced reduction in PDE activity. To evaluate this possibility we subjected MNL to Percoll separation and then recombined the 2 bands to produce the original MNL constitution. The PDE activity in HF-separated MNL was 0.13 pmol/min/10^6 cells, whereas the MNL that had passed through Percoll had their PDE activity reduced by 25% to 0.099 pmol/min/10^6 cells.

**Adherence Separation:** Adherence separation of monocytes increased the purity of those cells. Adherent cells were 94% monocytes in atopic preparations and 93% in normal preparations, as shown in Table I. These values were considerably higher than those obtained from the Percoll separation (Table I). We compared 4 AD subjects and 6 normal subjects (Table III).

Phosphodiesterase activity in MNL populations prior to adherence was significantly higher in AD patients compared with normal subjects. This difference between atopics and normals was much greater in the purified, adherent monocytes. The atopic PDE was 5-fold higher (0.207 ± 0.1 compared with 0.042 ± 0.02 pmol/min/10^6 cells) than in normal cells (p < 0.005). In addition, a significant but smaller elevation of PDE activity was seen in the nonadherent cells (p < 0.025), but these cells had considerably more contamination by monocytes in both normal (10%) and AD (9%) preparations and contained the majority of B cells. Similar 6-7% monocyte contamination in Percoll-prepared cells (Table I) may have been obscured by the observed reduction of PDE by Percoll separations and the lower B cell numbers.

**Adenylate Cyclase Activity** In spite of the marked elevation of atopic MNL PDE, the resting cAMP level does not differ from that of normal MNL [7]. We questioned whether the increased PDE was balanced by an elevation of AC activity. To investigate this possibility we measured the AC activity of MNL, MR, and LR fractions. Insufficient numbers of adherence-purified monocytes were obtainable for the adenylate cyclase assay. In both AD and normal preparations, the MR cells had basal AC significantly higher than that found in the LR fraction (Table IV) (p < 0.005). However, there were no differences in the AC activities between normal and AD cell preparations. Although the atopic MR fraction had a slightly higher AC activity, this was not statistically significant.

Forskolin, a catalytic unit stimulator of AC, was studied to evaluate stimulated enzyme activity in the various leukocyte preparations. Dose response studies of MNL showed optimal stimulation at 10^-3 M concentrations and this concentration was used in subsequent studies. We observed significant differences between basal and forskolin-stimulated AC activities. There were no differences in maximal AC activity between atopic and normal leukocyte preparations (Table IV). These AC studies utilized only homogenate preparations due to limitations on materials. In 2 cases where adequate cells were available, we compared AC activity in 106,000 g sedimented membrane preparations. Forskolin-stimulated values based on protein concentrations showed mean 7-fold increases in both membrane and homogenate activities, indicating that the latter provided reliable preparations for comparison.

**DISCUSSION**

This study confirms the elevated PDE activity in MNL from patients with AD. These data clarify our previous kinetic studies which showed kinetic similarities between the PDE with increased activity from AD MNL preparations and the PDE from normal monocytes.

Our results demonstrate that monocytes from patients with AD have elevated PDE activity when compared with normal control subjects. The difference between AD and controls was magnified when monocyte preparations of greater purity were obtained by adherence.

It is apparent from the data that preparative methods affect PDE activity and we have shown a 25% inhibitory effect of Percoll centrifugation on PDE activity. The similar values of PDE activity for nonadherent cells and MNL suggests a possible stimulatory effect on PDE activity during the 2-h adherence of MNL and it is clear that both AD and control samples were affected in a similar manner.

However, in spite of the differing effects of the 2 cell separation methods on PDE activity, both monocyte preparations from AD

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**Table II.** Cyclic AMP Phosphodiesterase Activity of Atopic Dermatitis (AD) and Normal Mononuclear Leukocyte Subsets

<table>
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<tr>
<th>Subset</th>
<th>AD (n = 9)</th>
<th>Normal (n = 8)</th>
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<tbody>
<tr>
<td>Mononuclear leukocyte</td>
<td>0.069 ± 0.006^a</td>
<td>0.026 ± 0.005</td>
</tr>
<tr>
<td>Monocyte-rich</td>
<td>0.052 ± 0.008^a</td>
<td>0.019 ± 0.005</td>
</tr>
<tr>
<td>Lymphocyte-rich</td>
<td>0.0067 ± 0.002</td>
<td>0.0062 ± 0.001</td>
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</tbody>
</table>

^a pmol/min/10^6 ± SEM.
^p < 0.005.
patients showed values significantly higher than those of control samples treated identically. Further, the results illustrate the importance of employing different methods of purification of mononuclear subsets when examining PDE activity. The PDE activity of AD lymphocytes compared with normals is less clear because lymphocytes obtained by the 2 preparative methods showed a significant difference by one method only. The LR fraction and the nonadherent cells both comprised lymphocytes predominantly and had lower PDE activity than the corresponding monocyte preparation. However, no difference between AD and normal lymphocytes was found in the 94% LR preparations, whereas the less pure nonadherent samples showed significantly higher PDE activity in AD cells.

Although the higher proportion of contaminating monocytes in the nonadherent lymphocyte preparations would be seen to be the initial explanation, the nonadherent cells appear to contain a higher proportion of B cells than the LR fraction. Normal B cells have been reported as having high PDE activity and although the PDE activity of B cells in AD is unknown at present, an elevated PDE has been implicated by the suppression of AD spontaneous IgE production by specific PDE inhibitors [20]. Thus, our data cannot exclude some elevation of lymphocyte, particularly B lymphocyte, PDE activity in AD.

Although atopic monocytes clearly have elevated cAMP metabolism, several studies have failed to demonstrate a difference in resting cAMP levels in leukocytes of normal and AD subjects [4,6,7]. This may indicate that the destruction of cAMP by PDE is balanced by increased production of cAMP by AD leukocytes. Our present data do not verify this proposal. We found no consistent or significant increase in either basal or maximal AC activity in any of the preparations, whether mixed MNL, LR, or MR. Unfortunately, the number of cells required for the AC assay precluded the use of a second method for preparing the leukocyte subsets to confirm the findings obtained on Percoll-purified preparations.

Abnormalities of AD monocytes have been implicated in several recent studies [21,22] and cyclic nucleotides may modulate several in vitro immune functions [23,24]. Herlin and Kragballe [21] associated impaired monocyte cyclic AMP responses with diminished monocyte cytoxicity in atopics. Our findings of increased PDE activity in this group of cells would satisfactorily explain their findings of diminished cAMP responsiveness. Impaired cyclic nucleotide metabolism in monocytes could affect a number of immune functions including phagocytosis, antigen presentation, and responses to lymphokines [25]. These in turn could account for some of the observed immunologic abnormalities in AD [1].

In summary, we have demonstrated that atopic monocytes have elevated PDE activity in the absence of a similar increase in resting or stimulated AC activity. Understanding the basis of cyclic nucleotide abnormalities in AD may allow clarification of the many pharmacologic and immunologic defects associated with atopic disease.

### Table IV: Basal- and 10⁻⁵ M Forskolin-Stimulated Adenylate Cyclase Activity of Atopic Dermatitis (AD) and Normal Percoll-Separated Mononuclear Leukocyte Subsets

<table>
<thead>
<tr>
<th></th>
<th>AD (n = 9)</th>
<th>Normal (n = 8)</th>
</tr>
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<tbody>
<tr>
<td>Mononuclear leukocyte</td>
<td>7.3 ± 1.5</td>
<td>7.6 ± 2.6</td>
</tr>
<tr>
<td>Lymphocyte-rich</td>
<td>9.0 ± 2.4</td>
<td>5.9 ± 1.6</td>
</tr>
<tr>
<td>Lymphocyte-rich</td>
<td>1.4 ± 0.5</td>
<td>2.3 ± 0.7</td>
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*pmol/min/10⁵ cells ± SEM.

**Comparison between basal- and forskolin-stimulated level.

### REFERENCES


