

Immunochemical characterization of the distinct monocyte cyclic AMP-phosphodiesterase from patients with atopic dermatitis

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Background: Previous findings have suggested that the immunopathology of patients with atopic dermatitis (AD) results from altered cellular responses caused by cyclic nucleotide regulatory abnormalities. One such defect is the increased degradation of the second messenger, cyclic adenosine monophosphate (cAMP), by elevated cAMP-phosphodiesterase (PDE) activity in patients with AD.

Methods: We used two monoclonal antibodies to identify the major PDE isoform in AD blood monocytes. We have also characterized the abnormal PDE activity by means of chromatofocusing and sucrose gradient centrifugation.

Results: The chromatofocusing technique allowed the separation of a PDE-containing fraction (isoelectric point = 6.1) from AD monocytes but not from normal cells. This monocyte fraction accounted for most of the elevated leukocyte-PDE activity and was a cytosolic, cAMP-specific, low Michaelis constant, calcium-calmodulin-dependent enzyme, inhibited by the cAMP-PDE inhibitor, Ro 20-1724. The majority of the PDE activity in this chromatofocused fraction was immunoadsorbed by the solid-phase immobilized antibodies against calcium-calmodulin-dependent PDE.

Conclusions: The increased degradation of cAMP by a unique form of PDE may cause defective regulation of intracellular functions of AD monocytes, leading to the characteristic hyperreactive immune and inflammatory events. Characterization of PDE isoenzymes from different leukocyte subpopulations may allow further expansion of cell-directed therapy for inflammatory disease. (*J ALLERGY CLIN IMMUNOL* 1993;91:1179-88.)

Key words: Atopic dermatitis (AD), chromatofocusing, immunoadsorption, mononuclear leukocytes (MNLs), phosphodiesterase (PDE), anti-PDE monoclonal antibodies

Peripheral blood mononuclear leukocytes (MNLs) of patients with atopic dermatitis (AD) manifest subnormal cyclic 3'-5' adenosine monophosphate (cAMP) responses to adenylate cyclase-activating agonists such as isoproterenol, histamine, and prostaglandins.¹⁻³ Previous studies

have shown abnormalities of immune function in AD, and these altered cellular responses may be consequent to defects in the cyclic nucleotide regulatory system. Though originally considered evidence for a defect of β -adrenergic receptors, the abnormality was found not to be receptor-dependent but due instead to increased degradation of cAMP by elevated cyclic AMP-phosphodiesterase (PDE) activity in atopic MNLs.⁴

The cyclic AMP-PDE enzyme system consists of many enzyme forms that catalyze the hydrolysis of cAMP. Four major isoenzyme families have been identified, all of which appear to relate to distinct units of a larger "supergene" family.⁵ These various isoforms are categorized in terms of molecular weight (MW), calcium-calmodulin dependency, differing substrate affinities as measured by Michaelis constant (Km) and modula-

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Abbreviations used

MNLs:	Mononuclear leukocytes
AD:	Atopic dermatitis
PDE:	Phosphodiesterase
MW:	Molecular weight
Km:	Michaelis constant
GBSS:	Gey's balanced salt solution
Hank's CMF:	Hank's calcium- and magnesium-free balanced salt solution
Ig +:	Flourescein-isothiocyanate-fluoresceinated polyvalent antihuman immunoglobulin-positive
EGTA:	Ethyleneglycol-bis-(β -amino-ethyl ether)-N,N,N ₁ ,N ₁ -tetraacetic acid
pI:	Isoelectric point
PBS:	Phosphate-buffered saline
ANAE:	Acid naphthyl acetate esterase
cAMP:	Cyclic adenosine 3',5'-monophosphate
cGMP:	Cyclic guanosine 3',5'-monophosphate
G/A:	Ratio of cGMP-PDE hydrolysis to cAMP-PDE hydrolysis
IC ₅₀ :	Fifty percent inhibition concentration
ACC:	Anti-calmodulin
ACAP:	Anti-calmodulin-dependent phosphodiesterase
CF:	Chromatofocusing

tion by cyclic guanosine 3',5'-monophosphate (cGMP). Our studies have focused primarily on a type IV low Km enzyme that shows high affinity for the cAMP substrate.^{4, 5} Most PDE studies have used nonhuman heart and brain tissue, and relatively little information exists for human isoforms.

Biochemical characterization of the PDE in atopic MNLs was done by means of chromatofocusing, which demonstrated two distinct, abnormal peaks in homogenates from patients with AD.⁶ These peaks appeared to represent enzyme isoforms in monocytes and lymphocytes. In the present study, we compared PDE activity from various subpopulations of leukocytes and confirmed monocyte PDE as the major abnormal PDE in AD. We next attempted to isolate and characterize this monocyte PDE form by means of chromatofocusing and enzyme kinetic study. From the characterization, we found atopic isoforms to be calcium-calmodulin-dependent. We used two monoclonal antibodies that identify the major calmodulin-dependent PDE isoform present in blood monocytes from patients with AD.

The identification of this PDE may allow further understanding of the regulatory mechanism involved in the leukocyte cAMP hyporesponsiveness to hormonal stimulation in AD. Consequent low cAMP levels in bone-marrow-derived cells can account for the abnormal immune and inflammatory events that are characteristic of AD. Better characterization of PDE isoenzymes may provide a biochemical marker for AD and could help the development of new PDE inhibitors for the treatment of atopic diseases.

METHODS

Materials

RPMI-1640 medium, Gey's balanced salt solution (GBSS), fetal bovine serum, neuraminidase, Hank's balanced salt solution (HBSS), Hank's calcium- and magnesium-free balanced salt solution (Hank's CMF) were obtained from Gibco (Grand Island, N.Y.). cAMP, imidazole, latex beads, acid naphthyl acetate esterase, (ANAE) and snake venom (5'-nucleotidase) were purchased from Sigma Chemical Company (St. Louis, Mo.). Hypaque-Ficoll, Percoll, polybuffer exchanger PBE74, polybuffer PB74, and the K 9/30 column were obtained from Pharmacia (Piscataway, N.J.). Tritiated cAMP (36 Ci/mmol), tritiated cGMP (20 Ci/mmol), carbon-14 methyl-labeled cytochrome C, ovalbumin, and bovine serum albumin (all with specific activity of 20 μ Ci/mg) were obtained from New England Nuclear (Boston, Mass.). Monoclonal antibody Leu 2b was from Becton-Dickinson (Mt. View, Calif.), and monoclonal antibody OKT4 was from Ortho Diagnostics (Raritan, N.J.). Purified rabbit complement was from Cedarlane Laboratories (Hornby, Canada). Resin AG1X2 (200-400 mesh), calmodulin (bovine heart) was obtained from Bio-Rad (Richmond, Calif.). Fluoresceinated, polyvalent antihuman immunoglobulins were from Hyland Laboratories (Costa Mesa, Calif.), and "Ready-Solv" scintillation fluid was purchased from Beckman (San Jose, Calif.).

Subjects

Normal healthy subjects who had no history of asthma, allergic rhinitis, or AD were selected for this investigation. Patients with active AD were chosen according to well-defined criteria,⁷ and all had moderate to severe AD. Donors received no systemic therapy for at least 96 hours before the study, and none had taken systemic or nasal adrenergic, PDE-inhibitor, or corticosteroid medications. No caffeine- or methylxanthine-containing beverages were consumed within 14 hours before the study. Venous blood was drawn into heparin-containing syringes (10 U/ml) for further processing.

Cell preparation

Mononuclear leukocyte isolation. Blood was separated by Hypaque-Ficoll gradient centrifugation at 400

g for 30 minutes.⁸ The cells were washed three times with saline solution and spun at 400 g, 300 g, and 250 g sequentially to eliminate platelet contamination. Microscopic visualization for leukocyte-adherent platelets, as well as Coulter counter (Coulter Corp., Hialeah, Fla.) platelet quantitation with standard settings, showed that the cell suspensions averaged less than 1% platelet contamination. Comparisons with Pawlowski's platelet depletion method in calcium-magnesium-free buffer⁹ yielded a similarly low value for platelet contamination of less than 0.5%. For differential lymphocyte and monocyte quantitations, Giemsa and ANAE stains¹⁰ and latex bead phagocytic ingestion were used. To enumerate phagocytic cells and B cell contamination in the monocyte-preparations, 10^5 cells in 75 μ l of RPMI/15% FBS were placed into a 6 $\times$ 50 mm glass tube, and latex beads of 0.8 μ m particle diameter were added at a final concentration of 0.3%, incubated for 45 minutes at 37° C with intermittent mixing, and then washed three times with PBS. To the resultant pellet, 75 μ l of fluoresceinated polyvalent anti-human immunoglobulins was added and incubated on ice for 30 minutes, washed, and observed under a microscope with fluorescence and phase-contrast illumination to determine percent nonphagocytic, fluorescent cells (B cells) and percent latex-ingesting cells (monocytes). These quantitations were monitored in all preparations and showed no differences between patients with AD and normal subjects in terms of percentages of monocytes and lymphocytes, which is consistent with our earlier report.¹¹

Monocytes. For routine monocyte adherence, Hypaque-Ficoll-separated MNLs were resuspended in RPMI 1640 medium with 15% human AB serum at 15×10^6 /ml and allowed to adhere at 37° C for 2 hours. Nonadherent lymphocytes were removed, and monocytes were scraped free with a rubber policeman and resuspended in Hank's CMF to minimize clumping. These preparations consisted of $96\% \pm 4\%$ phagocytic, ANAE-positive monocytes and $6.3\% \pm 2.1\%$ surface Ig+ cells. The platelet depletion method of Pawlowski et al.⁹ and Percoll methods yielded similar levels of monocyte purity,¹² and we observed no alterations in PDE activity with either method.¹¹

Nonadherent cells. After monocyte removal by routine adherence, lymphocytes were washed and reconstituted in Hank's CMF for further cell separation or in 0.1 mol/L imidazole for biochemical analysis. For T cell isolation, the nonadherent lymphocytes were mixed with an equal volume of 1% neuraminidase-treated sheep erythrocytes in the presence of 20% fetal calf serum. The mixture was incubated at 37° C for 15 minutes, spun gently at 200 g for 5 minutes, and then incubated for 1 hour at 4° C to allow rosetting. The rosetted cells were spun on Hypaque-Ficoll, and the interface (T-cell-depleted, B-cell-enriched) fraction was removed. The sheep erythrocyte rosette-forming cells in the pellet were gently resuspended and passed through Hypaque-Ficoll gradient again for greater pu-

rity (90% sheep erythrocyte rosette-forming cells in the pellet). The erythrocytes were then lysed with cold ammonium chloride (0.74%) buffer and the rosetting T lymphocytes were washed and resuspended in either GBSS or Hank's CMF, depending on experiments planned.

CD₄⁺- and CD₈⁺-enriched populations were obtained by cytolytic treatment of the T lymphocytes with the monoclonal antibodies Leu 2B and OKT4, respectively, and purified rabbit complement. Cell populations thus obtained were assayed for specific surface markers by fluorescence-activated cell sorter analysis, resulting in greater than 95% enriched populations.

Leukocyte homogenates

Freshly isolated mixed MNLs or purified lymphocyte or monocyte preparations were freeze-thawed three times in an acetone-dry ice bath and homogenized by a Dounce homogenizer (50 strokes) at 4° C. The resulting homogenates were centrifuged at 200 g to remove nuclear debris, yielding whole homogenates of nonnuclear membranous suspension. To provide soluble preparations for PDE determination, the whole homogenates were sonicated with a Braun sonicator (B. Braun Biotech, Allentown, Pa.) for 5 minutes at 30-second intervals (50 W) at 4° C. The solubilized portions of these whole homogenates were obtained by centrifugation for 1 hour at 4° C and 45,000 g in a Beckman L5-50B ultra-centrifuge (type 65 rotor). After removal of the insoluble debris, these sonicated, solubilized, whole homogenate preparations retained over 95% of the total PDE activity of the whole homogenates. These preparations were used for both chromatofocusing and sucrose gradient analysis.

To separate into membrane and cytosolic components, the unsonicated whole homogenate was spun at 40,000 g for 1 hour in a Sorvall RC-5B centrifuge (Du Pont Co., Wilmington, Del.). The resulting supernatant contained cytosolic PDE. The pellet of insoluble membrane was then rendered soluble by extensive sonication (5 minutes, 4° C, at 50 W) before chromatography. As noted above, this technique solubilizes >95% of total membrane PDE activity. Centrifugation at 45,000 g resulted in a similar cytosolic and membrane fractional distribution of PDE activity from normal and atopic MNL homogenates.

Phosphodiesterase assay

PDE was assayed with a method modified from that of Thompson et al.¹³ The incubation mixture (0.4 ml) contained 0.1 to 200 μ mol/L cAMP, 200,000 cpm of tritiated cAMP and 0.2 ml sample or standard PDE in 40 mmol/L Tris-C1 buffer (pH 8.0) that contained 3.75 mmol/L β -mercaptoethanol and 15 mmol/L MgCl₂. After incubation at 30° C for 10 minutes, the reaction was terminated by snap-freezing in an ethanol-dry ice bath, and the mixture was then boiled for 1 minute. Purified 5'-nucleotidase (0.45 U/ml) was added to the mixtures, which were then further incubated for 10

TABLE I. Cyclic AMP-phosphodiesterase activity in sonicated whole homogenates of various leukocyte fractions

	N	Normal (pmol/min/10 ⁶ cells)	AD (pmol/min/10 ⁶ cells)
MNLs	17	35 ± 11	71 ± 11*
Monocytes	17	42 ± 9	207 ± 12*
Total lymphocytes	17	36 ± 7	74 ± 17†
T cells	6	32 ± 17	73 ± 22†
CD ₄ ⁺	6	36 ± 13	58 ± 12‡
CD ₈ ⁺	6	22 ± 6	54 ± 21‡

Values are expressed as means ± SEM.

**p* < 0.001 compared with normal cell preparations.

†*p* < 0.01 compared with normal cell preparations.

‡*p* < 0.05 compared with normal cell preparations.

minutes at 30° C and then transferred to Pasteur pipette columns that contained AG1X2 resin. The radioactivity in the eluates was quantitated in scintillation fluid. Enzyme activity was expressed as unit = picomoles of cAMP hydrolyzed per milligram of protein. Protein concentration was determined by an assay with a BioRad protein dye. A standard PDE from bovine heart¹⁴ was used to monitor consistency and recovery in each assay. For comparison, the assay method of Beavo et al.¹⁵ yielded consistently similar results.

Cyclic GMP-PDE activity was determined by the procedures described above with 1 to 200 μmol/L ³H-cGMP as substrate (150,000 cpm). The G/A ratio of each fraction is defined as the rate of cGMP to cAMP hydrolysis a 1 μmol/L by the same amount of each enzyme fraction. This ratio provides an approximation of the substrate specificity of PDE activity in each fraction.

Chromatofocusing

Polybuffer exchanger PBE94 (Pharmacia) was saturated with 0.025 mol/L imidazole buffer (pH 7.4) and packed overnight in a K9/30 column (Pharmacia) at a linear flow rate of 100 cm · hr⁻¹. The void volume of the column was approximated with 1 ml of 2 mg/ml bovine cytochrome C (pI = 10.5). One milligram of solubilized protein was applied in polybuffer PB74 (1:10 dilution, pH 4) at 50 cm · hr⁻¹ flow rate over pH differences of approximately 0.05 between fractions, as previously described.⁶ Samples with similar pIs were pooled. The protein of each fraction was precipitated with saturated ammonium sulfate, redissolved, and dialyzed against GBSS. The samples were then lyophilized and reconstituted at 1 mg protein per milliliter in 40 mmol/L Tris (pH 8) before further assays were performed. Eighty-five percent to 93% of the applied enzyme activity was recovered in the pooled fractions.

Sucrose gradient centrifugation

With the use of a gradient former (BioRad model no. 385), 5% to 20% sucrose gradients were formed in

13 × 51 mm ultracentrifuge tubes (Beckman Ultraclear centrifuge tube). Membrane-free, solubilized whole homogenates (each with 1 mg protein/ml) were layered onto the gradients and spun at 160,000 *g* and 4° C for 19 hours in a Beckman L5-50B Ultracentrifuge with a SW65Ti rotor. Samples were collected from the bottom of the resulting gradients at 5 drops per fraction (approximately 200 μl per fraction). The ratio optical density 260/280 of each fraction was monitored by a Beckman spectrophotometer (Model DU-6). MW marker proteins used were ¹⁴C-methyl-labeled (20 μCi/mg) cytochrome C (MW = 12,300), ovalbumin (MW = 46,000) and bovine serum albumin (MW = 69,000). S units were estimated according to calculations by Martin and Ames.¹⁶ Linearity of each gradient was monitored by measuring the sucrose density of the collected fractions with a refractometer (Bausch and Lomb, model ABBE-3L). PDE activity, expressed as picomoles per minute per milliliter, was plotted against fraction number. Total PDE activity was estimated by calculating total area under gradient peaks.

PDE calcium-calmodulin dependency

Chromatofocused fractions for this study were first dialysed against a buffer that contained 40 mmol/L Tris at pH 8 and 50 μmol/L EGTA to remove remaining calcium and calmodulin. Each enzyme fraction was then assayed for PDE activity in the absence or presence of 50 μmol/L EGTA over a dose range of 0 to 150 μmol/L of CaCl₂, 0 to 20 μmol/L of calmodulin individually or with a mixture of calmodulin (5 μmol/L) and calcium (20 μmol/L) to give the ratio of 1:4 as established by Huang et al.¹⁷ In all experiments a control with polybuffer PB74 was tested to ensure no interference with the assay itself.

Anti-PDE monoclonal antibody quantitation of fraction B-PDE

Two murine monoclonal antibodies, anti-calmodulin-1 (ACC-1) and anti-calmodulin-dependent PDE (ACAP), directed against calmodulin-dependent PDE

were used. ACC-1 recognizes the calmodulin bound to PDE, and ACAP is directed against the PDE molecule when calmodulin is bound.¹⁸ Chromatofocused fractions were reconstituted after lyophilization to a concentration of 100 μ g protein per milliliter in 40 mmol/L Tris buffer at pH 8. A 20 μ l aliquot of each sample solution was used for monitoring the PDE activity before immunoadsorption. Antibody suspensions that contained either ACC-1 or ACAP were prepared as described previously.¹⁸ Prewashed *Staph A* cells (10%, wt/vol) were saturated by incubation of 1 mg ACC-1 or ACAP in 1 ml of *Staph A* suspension for 2 hours at 4° C. After washing, the *Staph A*/antibody complex (in 40 mmol/L Tris buffer, pH 7.5, 0.1% Tween and 150 mmol/L NaCl) was reconstituted in 10 times the original volume. Equal volumes (200 μ l) of antibody suspension and calcium-calmodulin buffer (500 μ mol/L calcium chloride and 10 μ g/ml calmodulin in a 40 mmol/L Tris buffer) were mixed and incubated overnight at 4° C. The immunoadsorbed complexes were then washed twice with a Tris-Tween buffer (40 mmol/L Tris buffer, pH 7.5; 0.1% Tween; and 150 mmol/L NaCl) and resuspended at 200 μ l per sample. Twenty microliters of the resulting suspension (enzyme-antibody-*Staph A* complex) was used for monitoring PDE activity. Results were expressed as percentage of total PDE activity or as picomoles per minute per milliliter absorbed to the solid-phase antibody complex.¹⁸ Nonspecific binding was evaluated with *Staph A* or *Staph A*-rabbit anti-mouse serum controls.

Statistics

Student's *t* test was performed to compare data obtained from normal donors and patients with AD.

RESULTS

Cyclic AMP-PDE activity distribution in leukocyte preparations

In all PDE activity determinations, a substrate concentration of 1 μ mol/L cAMP was used, and hence determinations refer to low *K_m* PDE. The enzyme activities in sonicated whole homogenates of various leukocyte fractions are listed in Table I. We found very high recovery of PDE activity with our separation techniques, even after further separation into cytosolic and homogenate fractions. The recovery profile for atopic MNLs (71 ± 11 PDE U/ml, *n* = 17, Table I) yielded 46.8 ± 5.7 U/ml in cytosol and 22 ± 8.8 U/ml in membrane fractions, a recovery of 96.9%. In pilot studies we assessed the possible effects of platelet contamination. Activity of normal MNLs separated by a fastidious platelet depletion method⁹ was 46 ± 8.9 U (mean $\pm$ SEM, *n* = 8) and not significantly different from the 35 ± 11 U (Table I) in the Hypaque-Ficoll gradient separated preparations, which were rendered free of platelets by sequen-

tial washing. Thus PDE activity was not higher in the cell preparations with the higher platelet counts, which confirms that platelet PDE contamination was insignificant in our leukocyte preparations.

In a comparison of cell types, PDE activity was quantitatively higher in both normal and AD monocytes than in lymphocytes. The most significant elevation was the five-fold higher PDE activity found in the atopic monocytes compared with normal monocytes (*p* < 0.001). The PDE activity in AD monocytes was 207 ± 12 pmol/min/ 10^8 cells, significantly greater than in the corresponding lymphocyte preparations (*p* < 0.001, Table I). This difference was not seen in comparisons of normal lymphocytes and monocytes (*p* = 0.086). The mean PDE activity in MNLs and lymphocytes from patients with AD was consistently about two times greater than that of the normal preparations, as were PDE activities in nonadherent AD lymphocytes and unseparated T cells. The PDE activity in the normal CD₄⁺ cells was higher, though not statistically higher, than that of the CD₈⁺ cells. AD CD₄⁺ cell PDE activity was similar to that of CD₈⁺ cells, and both activity levels were elevated compared with corresponding normal cells (*p* < 0.05). Because the preponderance of abnormal PDE activity in AD was localized to monocytes, we proceeded to evaluate and compare methods to characterize the monocyte PDE by means of sucrose gradient centrifugation and chromatofocusing column chromatography.

PDE forms

Our previous studies had shown that diethylaminoethyl chromatography failed to resolve atopic PDE fractions in MNL homogenates as clearly as did chromatofocusing,⁶ and we chose to use this technique primarily for separating monocyte PDE forms. Chromatofocusing resolved three major PDE-containing fractions (designated A, D, and F at pIs 6.7, 5.3 and 4.2, respectively) from sonicated, membrane-free, whole homogenates of normal and AD monocytes (Fig. 1) and additionally, a distinct, PDE-containing fraction (designated B) only from AD monocytes. These designations for the PDE-containing fractions (A, B, D, and F) represent the monocyte components of chromatofocused fractions. The lymphocyte PDE fractions of mononuclear leukocyte preparations, designated C and E, were previously shown to have distinct characteristics but considerably less activity.⁶

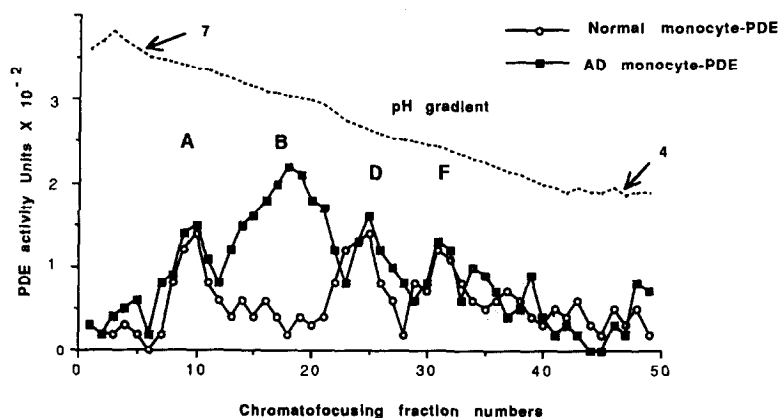


FIG. 1. Chromatofocusing separation of monocyte PDEs from paired experiments with normal and AD monocytes (eight pairs). One milliliter (1 mg) of solubilized whole homogenates was eluted over a pH gradient of 7 to 4. The fractions were then dialyzed, lyophilized, and reconstituted for PDE measurement. The pls of the recovered proteins were confirmed by isoelectric gel electrophoresis.

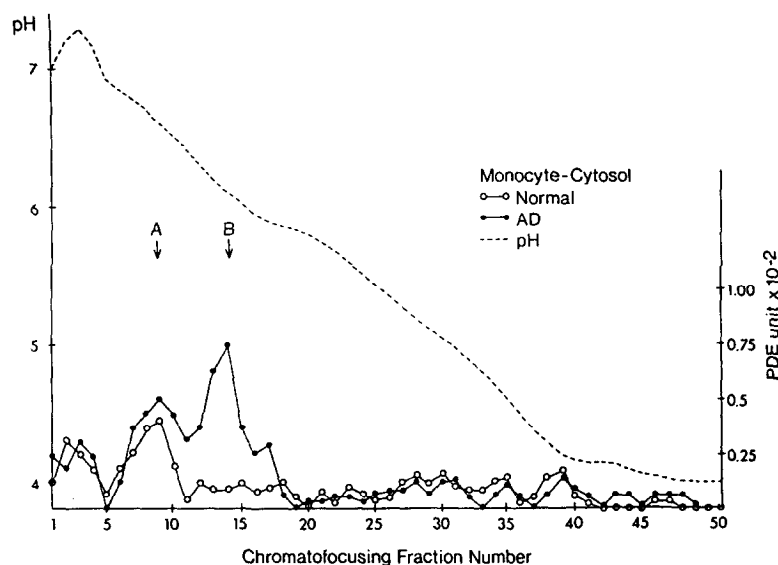


FIG. 2. Chromatofocusing separation of PDEs from the cytosol of the purified monocytes isolated from the blood of normal donors or patients with AD. The procedures in this experiment were identical to those of Fig. 3, except soluble cytosolic samples were used instead of sonicated, membrane-free, whole homogenates.

Unsonicated whole homogenates were further separated into cytosol and membranes before chromatofocusing. Fig. 2 shows representative chromatographs of PDE from normal and AD monocyte cytosol. The normal monocyte homogenate yielded only one major peak, fraction A, which showed PDE activity at pI 6.7. This constituted 76% of the cytosolic PDE activity, equivalent to 51% of the total activity recovered from monocyte whole homogenates. Two PDE-containing fractions (A and B) were isolated from AD monocyte cytosol with respective pIs of 6.7 and 6.1. These two fractions together contributed to

over 77% of the cytosolic PDE, equivalent to 61% of the total. Fraction A from normal and AD monocytes contained approximately equal amounts of PDE activity (145 pmol/min/fraction and 178 pmol/min/fraction, respectively). There was negligible PDE activity (<30 pmol/min/fraction) recovered from normal monocytes at pI 6.1 (fraction B). Thus the chromatofocusing technique yielded a distinctive PDE-containing fraction from atopic monocytes (232 ± 16 pmol/min/fraction).

The two activity peaks, D and F, observed in chromatographs of sonicated whole monocyte ho-

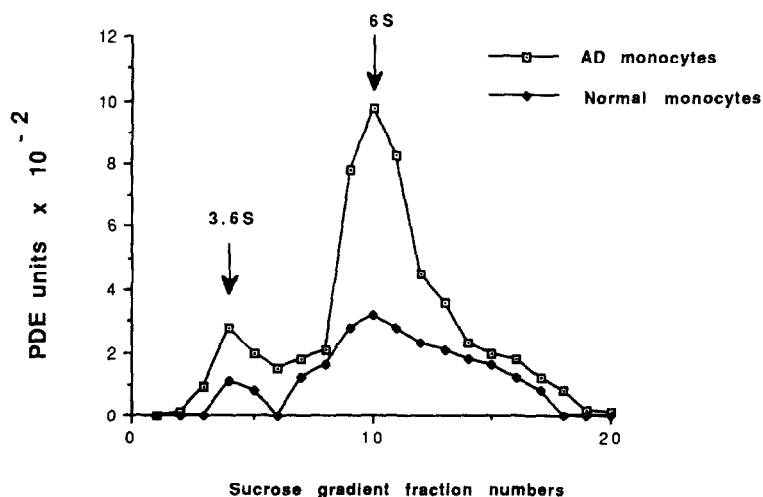


FIG. 3. Sucrose gradient centrifugation separation of PDEs from purified monocytes of normal donors and patients with AD ($N = 6$). Sonicated, solubilized whole homogenates at 1 mg protein per milliliter were layered onto the 5% to 20% sucrose gradients and spun at 160,000 g . The gradients were standardized with ^{14}C -labeled protein standards with known S values. The PDE-containing fractions were then dialysed and lyophilized; PDE activity was measured at 1 $\mu\text{mol/L}$ cAMP.

TABLE II. Characteristics of the PDE in chromatofocused fraction B of monocyte cytosol† from patients with AD

pI*	6.1
Km‡	0.89 $\mu\text{mol/L}$
G/A§	0.14
Calcium/calmodulin	$\uparrow 112\% \pm 37\%$
Ro 20-1724	$\text{IC}_{50} = 5 \times 10^{-6} \text{ mol/L}$

* S values and pIs were determined by sucrose gradient centrifugation and chromatofocusing as described in Methods.

†Cytosol and membranes were separated by 45,000 g centrifugation at 4° C for 30 minutes.

‡Km = Michaelis constant determined from the linear regression line established by Lineweaver-Burk plot; [cAMP] ranged from 0.1 to 200 $\mu\text{mol/L}$.

§G/A = the ratio of the rate of cGMP to cAMP hydrolysis by the same amount of enzyme from each fraction: [cGMP] = [cAMP] = 1 $\mu\text{mol/L}$.

||The effect of calcium and calmodulin was expressed as percent above basal activity. [cAMP] = 1 $\mu\text{mol/L}$ and [CaM] = 5 $\mu\text{mol/L}$.

mogenates (Fig. 1), were membrane PDE (data not shown) and showed no differences in either the PDE activity or pI between cells from normal and AD donors. This material was not further characterized.

We also assessed the use of sucrose gradient as a means of separating PDE isoforms in normal and AD monocytes. Fig. 3 shows sucrose-gradient-resolved PDE activity from sonicated solubilized whole homogenates (1 mg protein per milliliter gradient) of normal and AD monocytes. These gradients yielded 3.6S and 6.0S PDE-containing fractions. The PDE activity in the 6.0S fraction of the atopic monocytes was approximately three times that of the normal monocytes, whereas in the 3.6S fraction, it was considerably lower but was two times that of normal monocytes. Each fraction contained both normal and

AD enzyme activity, and neither of the fractions consisted of AD PDE exclusively. Recovery of PDE activity was $82.5\% \pm 7.2\%$ for experiments with AD monocytes ($N = 4$) and $78.7\% \pm 2.7\%$ for those with normal monocytes ($N = 7$), but each gradient required 1 mg of protein, and further resolution of the PDE in 6S or 3.6S fractions would have necessitated use of unacceptably great quantities of protein.

Characteristics and enzyme kinetics of fraction B-PDE from AD monocyte cytosol

The biochemical characteristics of chromatofocusing fraction B-PDE activity are summarized in Table II. As determined by pH gradient obtained from chromatofocusing, B-PDE comigrates with proteins at pI 6.1. Nondenaturing isoelectric focusing electrophoresis confirmed a PDE-contain-

TABLE III. Immunoabsorption of PDE in CF-separated fraction B from AD monocytes

	ACC-1 (4)	ACAP (6)
Monoclonal antibody (N)		
Immunoabsorption (% total)	57 ± 12.8	73.5 ± 12.8
PDE activity (pmol/min/ml)*	82.6 ± 15.6	148 ± 33.9

N, Number of experiments; % total, (immunoabsorbed PDE activity)/(total PDE activity).

*Substrate concentration was 1 μ mol/L cAMP; preimmunoabsorption PDE activity = 144.9 ± 17.6 pmol/min/ml for experiments with ACC-1 and 201 ± 36 pmol/min/ml for experiments with ACAP.

ing band at pI 6.1 when the AD monocyte cytosol proteins were analyzed. Activity was only present in the cytosol and not associated with the insoluble membranous preparations. It was a highly cAMP-specific enzyme with a G/A ratio of 0.14, and a low K_m of 0.89 μ mol/L cAMP. After pretreatment with EGTA, addition of calcium-calmodulin (4:1 ratio) activated the PDE activity to over 100% above basal level. In agreement with the G/A ratio, fraction B-PDE was sensitive to inhibition by the cAMP-PDE-specific inhibitor, Ro 20-1724, at 50% inhibition concentration (IC_{50}) = 5×10^{-6} mol/L assayed at 1 μ mol/L cAMP.

Antibody identification of the chromatofocusing fraction B-PDE

Because of the evidence that the AD monocyte-derived chromatofocusing (CF) fraction B-PDE was a calmodulin-activated enzyme, we further isolated this PDE by immunoabsorption with the use of two monoclonal antibodies directed against calmodulin-dependent PDEs, ACC-1 and ACAP.¹⁸ An average of 73.5% ± 12.8% (equivalent to 148 ± 33.9 pmol/min/ml of fraction, $N = 6$) of the AD monocyte CF fraction-B PDE activity was immunoabsorbed by ACAP (Table III). Similarly, ACC-1 immunoabsorbed 57% ± 12.8% (82.6 ± 15.6 pmol/min/ml, $N = 4$) of the PDE activity from identically prepared AD monocyte samples. Treatment with both ACC-1 and ACAP sequentially removed no additional immunoabsorbed activity. As described above, the CF fraction B of normal monocytes contained negligible PDE activity, and these findings are further confirmed by lack of immunoabsorption by PDE antibodies (21.7% ± 12.3% by ACAP and 18.6% ± 7.8% by ACC-1; $N = 6$ and 4, respectively, comparable to adsorption by rabbit anti-mouse control serum [17.8% ± 8.5%, $N = 12$]); that is, the PDE in fraction B monocytes accounted for the majority of the difference between normal PDE activity and that of the AD

cells. Control was also provided by studies of normal and AD lymphocyte fractions, which were likewise negligibly adsorbed by the monoclonal antibodies.

DISCUSSION

Unstimulated, purified monocyte preparations, isolated from either normal donors or patients with AD, showed significantly higher cAMP-PDE activity than the various purified lymphocyte preparations. This was observed regardless of whether the PDE activity was expressed in units per million cells or per milligram of protein; that is, the difference between AD and normal PDE was not a function of variations in monocyte protein synthesis. Elevated atopic monocyte PDE activity was not due to platelet contamination. Using the most careful platelet removal technique, we found no decrease in the PDE activity.

The atopic monocyte PDE is characterized by low K_m for cAMP, which has been termed PDE-IV.¹⁹ Evidence for cAMP specificity and selectivity includes a low G/A ratio and a low IC_{50} for compound Ro 20-1724. We have shown that this specific cAMP-PDE inhibitor and other newer agents have a preferentially greater effect on AD monocyte PDE activity.^{20, 21} This AD isoenzyme appears to be uniquely sensitive to inhibition, and it may serve as a marker for the atopic diathesis.²² The present report describes the initial studies aimed at characterizing and ultimately isolating the atopic monocyte PDE. Our chromatofocusing studies showed that both normal monocytes and monocytes from patients with AD contain a cytosolic PDE with pI of 6.7 and two membrane PDEs with pIs of 5.3 and 4.2 (Fig. 2). In addition, AD monocytes exhibit a cytosolic PDE with pI 6.1 (fraction B). Sucrose gradient separation showed that the majority of the increased PDE activity in AD monocytes was located in the 6S fraction, suggesting that the cytosolic PDE in fraction B corresponds to this fraction.

Because of our evidence that the low K_m

monocyte PDE activity from patients with AD was calcium-calmodulin-dependent, we proceeded to identify it with two monoclonal antibodies, which are directed against calmodulin-dependent PDE. ACC-1 directly recognizes calmodulin when bound to bovine heart PDE. ACAP is directed against the calmodulin-dependent PDE molecule when calmodulin is attached.¹⁸ Both antibodies significantly immunoadsorbed the PDE from the cytosolic preparations and from CF-purified fractions of AD monocytes. The finding of less than complete immunoprecipitation may indicate that the atopic isoform is quite distinct from the bovine brain PDE to which the antibodies were originally generated.¹⁸ The twofold calmodulin-activation of the atopic enzyme is also less than the activation often observed with other calmodulin-dependent PDE forms. Although these antibodies were originally directed against calmodulin-dependent PDEs with higher K_m values,²³ they confirm that the AD monocyte PDE is calmodulin-dependent. It may be possible to use these antibodies to further purify the low K_m , calmodulin-sensitive PDE for generation of a more specific antibody, leading to improved immunodetection sensitivity for enzyme present in AD cells. These antibodies indicate the potential for more efficient epidemiologic and genetic screening of individuals at risk for atopic diathesis and possible usefulness in purifying and characterizing leukocyte PDE. B-fraction PDE is cytosolic only. It is possible that an inactive, membrane-bound PDE was activated by a covalent mechanism such as phosphorylation or calmodulin binding, rendering it soluble and immunologically detectable by conformation-directed antibodies.

The increased PDE activity in atopic monocytes explains the previously described cAMP hyporesponsiveness to β -adrenergic stimulation^{1-3, 24} and accounts for functional abnormalities that have been observed in AD.^{25, 26} For example, increased basophil histamine releasability correlates with high PDE activity and is normalized after exposure of leukocytes to the PDE inhibitor, Ro 20-1724.²⁵ PDE also appears to play a role in spontaneous IgE synthesis by cultured B lymphocytes from patients with AD. Abnormally increased IgE synthesis was significantly reduced by preculture of cells in Ro 20-1724.²⁶ The net permissive effect of low cAMP consequent to increased PDE activity in bone-marrow-derived cells may account for many of the abnormally hyperresponsive immune and inflammatory events that characterize atopic

disease. The potential for use of new and more potent inhibitors of PDE-IV in the treatment of AD and respiratory allergy emphasizes the need for better characterization of PDE isoenzymes from other cells related to inflammation.

We have found no evidence for fluctuations of PDE activity relating to disease severity. In our original study we noted very high PDE levels in patients whose disease was in complete remission.⁴ Holden and Yuen²⁷ likewise found no relationship between PDE activity and extent or severity of patients' dermatitis. They noted roughly equal numbers of patients whose enzyme activities increased or decreased during 1-week treatment courses with topical fluorinated corticosteroids.

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