Interleukin-8–Stimulated Polyphosphoinositide Hydrolysis in Human Peripheral Blood Lymphocytes

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We have attempted to provide evidence for the production of inositol phosphate (IP) metabolites as an indication of specific receptor-mediated signal transduction in human peripheral blood lymphocytes (PBL) in response to interleukin-8 (IL-8). IP metabolites were measured, after loading of PBL with [3H]-D-myo-inositol, by anion exchange high-performance liquid chromatography (HPLC) and liquid scintillation counting of collected fractions. In addition, inositol 1,4,5-trisphosphate (IP3), in extracts from unlabeled cells, was measured using a specific radioligand binding assay. Compared with phytomagglutinin (PHA), which stimulated an increase in IP metabolites and, specifically, IP3 by greater than threefold, human recombinant (hr) IL-8 (1 nmol/L) also stimulated an increase in IP metabolites, as measured by HPLC, and a greater than threefold increase in IP3. The increase in IP3 was observed as early as 15 seconds after stimulation with hrIL-8, reaching maximal levels by 30 seconds. To further assess the signal transduction mechanism involved, the protein tyrosine kinase inhibitor genistein was added to the cells 10 minutes before stimulation with hrIL-8. After preincubation of PBL with this inhibitor, the generation of IP3 in response to PHA (5 μg/ml) and hrIL-8 (1 nmol/L) was inhibited by 42% and 51% of control values, respectively. In contrast to the production of IP metabolites, there were only small increases in intracellular calcium in response to hrIL-8 when compared with PHA.

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RECEPTOR STIMULATION in a large variety of cell types and in response to a diverse array of soluble mediators often results in the hydrolysis of the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2). This reaction leads to the generation of inositol-1,4,5-trisphosphate (IP3) and 1,2-sn-diaacpglycerol (DAG), which mobilize intracellular calcium and activate protein kinase C (PKC), respectively. However, the initial increase in intracellular calcium induced by IP3 is transient and the levels are subsequently maintained by transmembrane flux through either voltage-dependent, receptor-operated, or second messenger-operated calcium-mobilizing channels.1,3

We have previously shown that the migratory response of human peripheral blood lymphocytes (PBLs) to human recombinant interleukin-8 (hrIL-8) was inhibited by voltage-dependent calcium channel antagonists, was PKC-dependent, as shown by inhibition using PKC inhibitors, and that the activation of G-proteins was likely to be involved, as shown by studies using cholera and pertussis toxins.4,5

Therefore, the signaling events involved in hrIL-8–induced PBL migration may involve specific receptor-mediated opening of calcium channels induced by, or concomitant with, PKC activation. Recently, it has become evident that G-proteins may directly couple phospholipase C to calcium-mobilizing receptors, and that the activity of this enzyme may be regulated by tyrosine phosphorylation.6,7 Therefore, we have investigated the changes in the concentrations of IP1, intracellular calcium, and the effect of the specific protein tyrosine kinase (PTK) inhibitor genistein7 in hrIL-8–stimulated PBLs. In the present study in which the intracellular dyes Fura-2-AM and Indo-1-AM were used, only small detectable changes in intracellular calcium in response to hrIL-8 were obtained in human PBLs or the human T-cell leukemia cell line, Jurkat, when compared with phytomagglutinin (PHA) as a control. However, a substantial increase in inositol phosphates was seen in response to both stimuli. These novel observations indicate that hrIL-8–stimulated PBL migration may be associated with rapid elevations in the levels of IP3, but also with small increases in intracellular calcium.

MATERIALS AND METHODS

Materials. Materials for preparation of mixed human PBLs were obtained as previously described.8 Medium 199 (low inositol), ethylene glycol-bis (β-aminoethyl-ether) N,N,N',N'-tetraacetic acid (EGTA), Tris base, digitonin, dimethyl sulfoxide (DMSO), Earle's balanced salt solution (EBSS), LiCl, ammonium phosphate, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), bovine serum albumin and PHA (from Phaseolus vulgaris; PHA-L), propidium iodide, and Fura-2AM were obtained from Sigma Chemical Co (Poole, Dorset, UK). [3H]-D-myo-inositol (specific activity 18.9 Ci/mmol) and the [3H]-D-myo-inositol trisphosphate radioligand binding assay were purchased from Amersham International plc (Buckinghamshire, UK). Chloroform and methanol were obtained from Fisons Laboratory Supplies Ltd (Loughborough, UK), and hrIL-8 was obtained from Peprotech (Princeton Business Park, Rocky Hill, NJ). This recombinant protein was derived from Echerichia coli and greater than 98% pure, as assessed by N-terminal sequencing. The endotoxin level of the protein was less than 0.1 ng/μL. NaCl, KCl, MgCl2, CaCl2, glucose, and KOH were all obtained from BDH Ltd (Dagenham, Essex, UK). Genistein was obtained from ICN Biochemicals (Division of ICN Biomedicals Ltd, Cleveland, OH). Indo-1-AM (1-[2-amino-5-(6-carboxyindol-2-yl)-phenoxy]-2-[2-amino-5-methylphenoxy]-ethane-N,N,N',N'-tetraacetic acid) was obtained from Calbiochem Corp (Lucerne, Switzerland).

PBL preparation. Mixed human PBLs were obtained as previ-
ously described. Purified PBLs contained less than 1% monocyes, as assessed by immunocytochemical staining.

Jurkat cells were generously provided by Dr D. A. Cantrell (Imperial Cancer Research Fund Laboratories [ICRF], London, UK), and were maintained in RPMI 1640 supplemented with 25 mmol/L HEPES, 5% heat-inactivated fetal calf serum (HIFCS), 2 mmol/L L-glutamine, and 50 μg/mL gentamycin.

Measurement of phosphatidylinositol metabolism. PBLs were resuspended at a concentration of 5 × 10^6 mL in HEPES-buffered Medium 199 containing 10% dialyzed HIFCS, gentamycin (5 μg/mL), and 5 μCi/mL. [3H]-myo-inositol for 72 hours at 37°C. After 72 hours, the cells (>95% viable) were pelleted and the supernatant retained for the measurement of radiolabeled uptake. PBLs were then washed twice in EBSS containing 5 mmol/L LiCl and aliquots containing 2 to 5 × 10^6 cells in 250 μL EBSS/LiCl were allowed to equilibrate at 37°C in 5% CO2 before assay. Samples were then challenged with PHA, hrIL-8, or phosphate-buffered saline (PBS) for 10 minutes. Extraction of inositol phosphate (IP) metabolites was performed after acidification of the cell suspension with 1 mol/L HCl, by using chloroform/methanol/cell suspension (1:10:8, vol/vol in a total of 1 mL). The upper, aqueous phase was then neutralized (0.1 mol/L NaOH) before analysis by high-performance liquid chromatography (HPLC).

HPLC analysis of IP metabolites was performed according to the method of Dean and Moyer. Aliquots (200 μL) of sample supernatant were analyzed by anion exchange HPLC using a Partisil 10 SAX, 4.6 mm × 25 cm column and precolumn containing anion exchange pellicular packing (Whatman International Ltd, Maidstone, UK). The column was eluted with step-wise gradients of water/ammonium phosphate (pH 3.8) according to the following protocol: 0 to 30 minutes, 0.01 to 0.08 mol/L; 30.01 to 60 minutes, 0.2 to 0.28 mol/L; 60.01 to 90 minutes, 0.5 to 0.52 mol/L using an Automated Gradient Controller (Millipore Corp, Waters Chromatography Division, Hertfordshire, UK) at a flow rate of 1 mL/min. One-minute fractions were collected and aliquots (200 μL) were mixed with 1 mL (fractions 1 through 65), or 3 mL (fractions 66 through 90) of Optiphase Hisafe II scintillation fluid for counting (5 min/sample) in an LKB 1209 Rackbeta counter (Pharmacia LKB, Uppala, Sweden). AMP, ADP, and ATP were used as reference standards (259-nm wavelength) for inositol-4-monophosphate (I(4)P), inositol-1,4-bisphosphate (I(1,4)P), and inositol-1,4,5-trisphosphate (I(1,4,5)P), respectively, as described.

In separate experiments, purified PBLs were stimulated with PHA or hrIL-8 over a time course of 0 to 30 minutes in the absence of [3H]-myo-inositol. Assay for IP3 was performed using the Amersham D-myo-inositol-1,4,5-trisphosphate [3H] assay kit, according to the manufacturer’s instructions. In similar experiments, PBLs were preincubated for 10 minutes with genistein (30 μg/mL). Cells were then washed in EBSS/LiCl and challenged for 10 minutes with either PHA or hrIL-8 and IP3 levels were measured as described above. All results are expressed as mean ± SEM for the number of experiments shown.

Measurement of intracellular calcium concentration. Measurement of changes in intracellular calcium concentration was performed according to standard methods. PBLs or Jurkat T cells were loaded with Fura-2AM (1 mmol/L final concentration) at a concentration of 0.75 × 10^7 cells/mL in MEM containing 10% HIFCS for 30 minutes at 37°C. After loading, cells were resuspended in hydrolysing buffer (Solution A: 140 mmol/L NaCl, 5 mmol/L KCl, 1.8 mmol/L CaCl2, 0.9 mmol/L MgCl2, 25 mmol/L glucose, 6 mmol/L Tris base, and 10 mmol/L HEPES) for 30 minutes at 37°C. After hydrolysis of the Fura-2AM, cells were washed in solution A and maintained at 37°C before assay. For assay of calcium mobilization, cells in suspension (2 × 10^6/mL) were maintained at 35°C to prevent excessive leakage of Fura dye, and were stimulated with increasing concentrations of either PHA or hrIL-8. Changes in fluorescence were measured using a Perkin-Elmer LS-5B luminescence spectrophotometer (Perkin-Elmer, Hertfordshire, UK), with excitation and emission wavelengths being 346 and 492 nm, respectively. In separate experiments, human PBLs were labeled for 30 minutes to 1 hour with 0.5 μg/mL Indo-1 AM, in medium RPMI-1640, containing 10% HIFCS, L-glutamine, and antibiotics. Washed cells were resuspended in medium containing 10% HIFCS at a concentration of 10^6/mL and maintained at 37°C for fluorescence-activated cell sorter (FACS) analysis. Calcium flux was measured by modifications of the methods of Alexander et al14 using a FACStarPLUS flow cytometer (Becton Dickinson, Erembodegem, Belgium) equipped with a 5-W argon ion laser (Spectra Physics, Basel, Switzerland) set to UV emission (150 mW, 351 to 364 nm multiple lines). Filters for the detection of Indo-1 emission were FL4 (calcium-bound Indo-1), collected using a 405/10 bandpass filter, and FL3 (free Indo-1), collected using a 485/22 bandpass filter. The ratio of FL4 over FL3 (FL4-R), which is proportional to the intracellular calcium concentration, was computed in real time for each event using the pulse processing module of the cytometer. Gates were set to exclude dead cells after addition of propidium iodide, and changes in calcium flux were analyzed over 10 minutes for each experiment.

RESULTS

HPLC analyses of PHA- and hrIL-8-stimulated IP metabolism. Figure 1 shows the results obtained by HPLC analysis of PHA- (5 μg/mL; n = 3) and hrIL-8- (1 nmol/L; n = 4) stimulated human PBLs. Under the conditions used (72 hours, in the presence of radiolabeled inositol), it was found that approximately 5% to 8% of the [3H]-inositol added was metabolized in the PBLs, the remainder eluting very early on during the HPLC run (3 to 4 minutes; data not shown). Figure 1 shows the amount of radioactive IP3, and its metabolites IP2 and IP1, generated in response to both PHA and hrIL-8.
(D0Xn) W L-En IC) W A n-l (D F n-En a-Wr) B700 600 500 400 300 200 100 0

Fig 2. (A) The total IP_3 generated in response to PHA and hrlL-8. Using the Amersham radioligand binding assay, the concentration of IP_3 after a 10-minute challenge with either PHA (n = 8) or hrlL-8 (n = 9) has been calculated. Results represent the mean ± SEM pmol IP_3 per 5 × 10^6 cells. (B) The time course of generation of IP_3 in response to 1 nmol/L hrlL-8. Each point represents the mean ± SEM concentration per 2 × 10^6 cells, from four experiments.

and hrlL-8 (not seen with PBS control; results not shown; n = 4). After stimulation with either agonist, the majority of radiolabel was recovered in the fraction eluting in the position of glycerophosphoinositol^ and that co-eluting with ADP, indicating the presence of I(1,4)P_2.

Measurement of IP_3 by radioligand binding assay. The direct IP_3 binding assay was also used to quantify the production of this metabolite, after challenge with PHA (5 μg/mL) or hrlL-8 (1 nmol/L). Figure 2A shows the response obtained to PHA and hrlL-8 using this assay, following 10 minutes of stimulation with agonist. In response to PHA (n = 8), the resting (control) concentration of IP_3 was increased more than threefold. Similarly, hrlL-8 stimulation increased the concentration of IP_3 by greater than threefold (n = 9). To establish the time course of generation of IP_3, in response to hrlL-8, the reaction was terminated at 15 seconds, 30 seconds, 1 minute, 10 minutes, and 30 minutes. As can be seen in Fig 2B, there was a rapid induction of IP_3, with levels already being increased by 15 seconds and reaching a maximum at 30 seconds (440 ± 160 pmol per 2 × 10^6 cells; n = 4).

Effect of genistein on IP_3 production. Figure 3 illustrates the effect of preincubation with the PTK inhibitor genistein (30 μg/mL) for 10 minutes on PHA- and hrlL-8-stimulated IP_3 accumulation in PBL. After preincubation, the concentration of IP_3 produced in response to PHA (5 μg/mL) and hrlL-8 (1 nmol/L) was completely reduced to control levels. In all experiments, the viability of cells was greater than 95%, as assessed by trypan blue exclusion.

Effects of PHA and hrlL-8 on intracellular calcium mobilization. The effect of PHA on intracellular calcium concentration in PBL is well documented. In this study, PHA (5 to 40 μg/mL) induced a concentration-dependent increase in intracellular calcium in PBLs and Jurkat T cells, loaded with either Fura-2-AM (Fig 4) or Indo-1-AM. In contrast, using Fura-loaded cells no detectable increase in intracellular calcium concentration was observed in response to 0.1 to 100 nmol/L hrlL-8 (Fig 4E). This observation was consistent, irrespective of the concentration of cells used or the duration of calcium measurements on stimulated cells. Similarly, simultaneous or consecutive addition of hrlL-8 (1 nmol/L) and suboptimal concentrations of PHA (1 to 5 μg/mL),

Fig 3. The effect of genistein on PHA- and hrlL-8-induced IP_3 generation. After a 10-minute preincubation of PBLs with 30 μg/mL genistein (m), cells were washed twice and challenged for 10 minutes with either PHA (5 μg/mL, n = 3) or hrlL-8 (1 nmol/L, n = 4). Control shows IP_3 generation in the absence of PHA, IL-8, or genistein (n = 4). Results represent the mean ± SEM values.
Fig 4. Calcium flux in normal human PBLs in response to PHA (1 to 40 μg/mL; A through E) and hrIL-8 (F; 10^-8 M [i], followed by 10^-8 mol/L [ii] and 1 μg/mL PHA [iii]). Cells were loaded with Fura-2-AM and calcium mobilization measured by fluorimetry as described in Materials and Methods. Arrows represent the time of addition of the stimulus. Data are represented as fluorescence changes proportional to [Ca^{2+}] against time (minutes) and are representative of n = 6 experiments.

showed no additive or synergistic increases, or decrease in calcium flux over that observed with the corresponding concentration of PHA alone (Fig 4E). However, when cells were loaded with Indo-1-AM there was a small detectable increase in calcium flux in response to hrIL-8, as measured by FACS analysis. Figure 5 shows the results obtained on normal human PBLs in response to 10^-8 mol/L hrIL-8 in comparison with (inset) PHA at 5 μg/mL. Whereas there was a rapid flux of calcium in response to PHA, with greater than 50% of cells responding, the flux in response to hrIL-8 was slow, lasting up to 15 minutes, and of much smaller magnitude (<100 nmol/L compared with 400 nmol/L increase with PHA), with less than 10% of cells responding. In both cases, while there was a decrease in responding cell intracellular calcium concentration, there was never a complete reversal to resting levels. There was no greater increase in intracellular calcium concentration observed in response to higher concentrations of hrIL-8, and no change from resting levels observed on stimulation with PBS alone (n = 6, data not shown).

**DISCUSSION**

Although IL-8 is well known to induce PBL responses in vitro, clear evidence for IL-8 receptors on PBLs has yet to be reported. We have previously hypothesized that this inflammatory cytokine acts on PBL by direct receptor stimulation, in the light of possible calcium channel activation, stimulation of PKC, and putative involvement of G-proteins. The results presented here support this hypothesis by showing the hrIL-8-induced production of inositol metabolites in PBLs and their regulation by genistein.

The results of the HPLC analyses showed that less than 10% of radioactivity represented IP metabolites. In studies on thyroid-releasing hormone-stimulated GH3 cells, go

FACS analysis and variations on the HPLC system used in the present study, similar levels of IP metabolites were observed.
hrIL-8 induced a rapid increase in IP₃ production, maximal levels occurring by 30 seconds, following closely the profile of PHA-stimulated IP₃ production (K.B.B., D.G.Q., unpublished observations, July 1990).

To further characterize the possibility of a receptor-mediated IL-8 response, the PTK inhibitor genistein was added to the PBLs for 10 minutes before stimulation of cells with PHA and hrIL-8. In our experiments, there was a decrease in the generation of IP₃ in response to PHA and hrIL-8. The results with PHA are similar to those measuring the IP metabolites in the experiments of Mustelin et al., where, essentially, complete inhibition was observed. Once again, the effects of PHA and hrIL-8 were similar in terms of metabolite concentration produced and level of inhibition. Genistein, while being used in many studies for inhibition of protein tyrosine kinase, inhibits a number of tyrosine kinases, and certain tyrosine kinases are thought to regulate PI-specific phospholipase C (PL-C). Because the nature of the IL-8 receptor on human lymphocytes has not been characterized, it is impossible to determine precisely the tyrosine phosphorylation events leading to generation of IP metabolites in response to hrIL-8 in human PBL without a more complete biochemical study. However, as tyrosine phosphorylation of PL-C may be required for full enzyme activity, we could postulate that PL-C is a substrate for a putative tyrosine kinase associated directly, or indirectly, with the IL-8 receptor.

The mitogen PHA has long been recognized to stimulate the mobilization of intracellular calcium in human T cells. In our experiments, we consistently detected increases in intracellular calcium concentration in response to PHA, as well as increases in inositol metabolite production. However, in contrast to these results it was consistently difficult to obtain detectable changes in intracellular calcium concentration in more than 10% of the cells, in response to hrIL-8, in either PBLs or Jurkat T cells despite an increase in IP₃ levels comparable with that seen with PHA. While this number of responder cells is low, this correlates closely with the number of cells responding to hrIL-8 in in vitro chemotaxis assays (K.B.B., unpublished results, September 1989). This also contrasts with the situation in human PMN where a rapid increase in intracellular calcium is seen in response to IL-8. This may be caused by the insensitivity of the assay...
used to measure intracellular calcium, although this is hard to reconcile with the calcium mobilization seen in response to PHA and the approximately equivalent levels of IP metabolites obtained in response to both agonists. It has been suggested that there may be a threshold level of IP required for induction of calcium release from intracellular stores.\textsuperscript{30} However, hrIL-8 failed to increase the concentration of intracellular calcium when added to cells together with suboptimal concentrations of PHA. It is possible that the IP\textsubscript{3} produced in response to hrIL-8 is compartmentalized and therefore not available for liberation of intracellular calcium to the same extent as with PHA. Recently, it has been shown that muscarinic or serotonergic stimulation of cultured sympathetic neurones fails to increase cell body intracellular calcium when added to cells together with suboptimal concentrations of agonists.\textsuperscript{31} We have previously demonstrated the inhibition of hrIL-8–induced PBL migration by voltage-dependent calcium channel antagonists,\textsuperscript{9} suggesting a requirement for calcium influx in hrIL-8–mediated PBL responses. However, the presence of voltage-dependent calcium channels in human lymphocytes is disputed,\textsuperscript{32,33} therefore it is unclear as to what function the specific antagonists were having in our system. One cannot rule out the activation of specific ion channels by either IP\textsubscript{3},\textsuperscript{34} or direct G-protein activation,\textsuperscript{22} which may have been nonspecifically inhibited by these channel antagonists. Therefore, it is possible that the signal transduction mechanism involved in hrIL-8–induced PBL migration is different than those commonly seen with mitogenic mediators and does not involve the rapid, transient increase in intracellular calcium. By its very nature, the migratory response is a continual process of change in the cell microfilament network, implying the need for sustained levels of intracellular signalling components. Because we have shown a prolonged increase in IP\textsubscript{3} in response to hrIL-8 and investigations have demonstrated direct coupling of components of the phosphatidylinositol cycle with the motile apparatus and structural proteins of cells,\textsuperscript{35–38} it is possible that the motility of human PBLs in response to hrIL-8 is independent of rapid, transient, and measurable increases in intracellular calcium.

These and our previous published results\textsuperscript{5,6} suggest that hrIL-8 may be acting through a specific receptor that is coupled to a phosphatidylinositol-specific PI-C in human PBLs. The exact nature of the receptor awaits further characterization.

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