

# Regulation of Nucleoside Transport by Lipopolysaccharide, Phorbol Esters, and Tumor Necrosis Factor- $\alpha$ in Human B-lymphocytes\*

(Received for publication, June 1, 1998, and in revised form, August 1, 1998)

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**Nucleoside transport systems and their regulation in human B-lymphocytes have been characterized using the cell lines Raji and Bare lymphoma syndrome-1 (BLS-1) as experimental models. These cells express at least three different nucleoside transport systems as follows: a nitrobenzylthioinosine-sensitive equilibrative transport system of the *es*-type, which appears to be associated with hENT1 expression, and two Na<sup>+</sup>-dependent transport systems that may correspond to N1 and to the recently characterized N5-type, which is nitrobenzylthioinosine-sensitive and guanosine-preferring. B cell activators such as phorbol 12-myristate 13-acetate and lipopolysaccharide (LPS) up-regulate both concentrative transport systems but down-regulate the equilibrative *es*-type transporter, which correlates with lower hENT1 mRNA levels. These effects are dependent on protein kinase C activity. Phorbol 12-myristate 13-acetate and LPS also induce an increase in tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) mRNA levels, which suggest that this cytokine may mediate some of the effects triggered by these agents, since addition of TNF- $\alpha$  alone can increase N1 and N5 transport activities by a mechanism that also depends on protein kinase C activation. Interestingly, TNF- $\alpha$  down-regulates *es* activity, but this effect cannot be abolished by inhibiting protein kinase C. This study reveals differential regulation of nucleoside transport systems following activation of human B-lymphocyte cell lines by agents of physiological relevance such as TNF- $\alpha$  and LPS. Moreover, it indicates that the recently characterized N5 transport system can also be regulated following B cell activation, which may be relevant to lymphocyte physiology and to the treatment of lymphocyte malignancies.**

extent, in immature B cells (2). These actions are independent of cGMP, a second messenger in B cell activation (3). Moreover, nucleosides can mimic, both *in vitro* (4) and *in vivo* (5), a T cell-like signal for B cells that enables them to elicit antigen-specific responses to T cell-dependent antigens in the absence of T cells (6). These regulatory properties of nucleosides may be dependent on their uptake into the cell (1). Thus, the characterization of nucleoside transport systems and their regulation in these cell types may contribute to a better understanding of the role of nucleosides in lymphocyte physiology. Moreover, evidence that most antiviral and antiproliferative drugs used in lymphocyte malignancies can be substrates of these transport systems (7) provides additional stimulus in the attempt to identify the major routes for nucleoside uptake into lymphocytes and how these transport systems are regulated during B cell activation.

Several nucleoside transport systems have been described in mammalian cells (8). Two of them, *es* and *ei*, are equilibrative, show broad substrate specificity, and differ in their sensitivity to NBTI<sup>1</sup> inhibition. The former is inhibited by nanomolar concentrations of the analog, whereas the latter is barely inhibited at micromolar concentrations of NBTI. Two cDNAs, ENT1 and ENT2, have recently been isolated from rat and human tissues, and they appear to encode *es*- and *ei*-related proteins, respectively (9–12). Up to five concentrative Na<sup>+</sup>-dependent transport systems have been characterized kinetically in mammalian cells and classified from N1 to N5. N1, N2, and N3 correspond to well known transport agencies involved in purine-preferring, pyrimidine-preferring, and broad substrate specificity transport activities, respectively (8). The kinetic identity of N4 is somewhat controversial, whereas N5 is associated with a Na<sup>+</sup>-dependent NBTI-sensitive nucleoside transport activity that has been partially characterized in human leukemia cells and appears to be guanosine-preferring (8, 13). So far, only the human and rat counterparts of N1 and N2 related cDNAs have been cloned, named CNT2 (sodium purine nucleoside transporter) and CNT1, respectively (14–17). CNT1 and CNT2 are coexpressed in absorptive epithelia, liver, and, probably, brain (14–18). Coexpression of two isoforms in a single cell type may also involve isoform-specific regulation, as recently shown in liver parenchymal cells for the CNT1- and CNT2-related carrier proteins (19).

Little is known about the regulatory properties of nucleoside

Nucleosides and some of their metabolites trigger a variety of regulatory effects in biological systems. Indeed, guanosine derivatives exert immunostimulatory responses (1) and may trigger mitogenic effects in mature B-lymphocytes and, to a lesser

\* This work was supported in part by Fondo de Investigaciones Sanitarias Grant 96/2050 (to A. C.) and Dirección General de Investigación Científica y Técnica Grants PB92-0867 and PB95-0975 (to M. P. A.) and SAF-98/102 (to A. C.) and FAPS/Marató de TV3 contra el Càncer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ Supported by a fellowship from "Programa Ciência e Tecnologia do 21 Quadro Comunitário de Apoio", PRAXIS XXI Program, Portugal.

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<sup>1</sup> The abbreviations used are: NBTI, nitrobenzylthioinosine; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; BLS, Bare lymphoma syndrome; PCR, polymerase chain reaction; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide; BSDM, bisindolylmaleimide I; MOPS, 3-(*N*-morpholino)propanesulfonic acid; oligo, oligonucleotide.

transport in human lymphocytes and immune system cell types. Moreover, previous reports of changes in  $\text{Na}^+$ -dependent nucleoside transport associated with differentiation of HL-60 cells lacked a detailed kinetic analysis of the N-type transporters involved in such a response (20–22). This is in contrast with the detailed kinetic analysis reported in murine leukemia cells, showing that they express at least three transport systems for nucleosides, the two equilibrative *es*- and *ei*-types and a  $\text{Na}^+$ -dependent system that appears to be of the N1-type (purine-preferring) (23, 24). However, species-specific expression of carrier proteins is also likely on the basis of previous reports showing marked differences in nucleoside concentrative transport activity between human and mouse macrophages (25). Moreover, the contribution of the recently characterized N5 transport system may have been overlooked in previous studies involving lymphocytes and related cell types. Here we have identified the nucleoside transport systems expressed in human B cells, and we have determined how PMA, LPS, and  $\text{TNF-}\alpha$  can modulate these transport activities. PMA and LPS promote activation, differentiation, and proliferation in a variety of leukemic B cells (26–28). Despite some controversial data,  $\text{TNF-}\alpha$  may also be involved in B cell proliferation, by a probable autocrine loop which results in its release induced by LPS itself and by other activating agents (28–30). Evidence shows differential regulation of nucleoside transport by these agents in lymphocytes. Moreover, the N5-like activity found in these cell types is also modulated by LPS,  $\text{TNF-}\alpha$ , and PMA, which is the first report of regulation of the recently characterized N5 transport system.

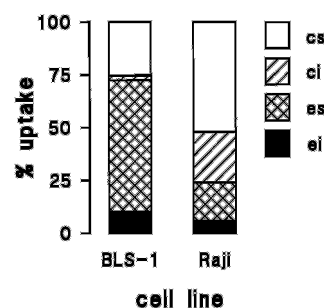
#### EXPERIMENTAL PROCEDURES

**Cell Lines and Culture Conditions**—Human B-lymphocyte cell lines Raji and BLS-1 were used in this study. Raji is a lymphoblast-like cell line derived from Burkitt lymphoma which is cultured in suspension. This cell line has been routinely used as a model of human B-lymphocytes. BLS-1 is a parental B-lymphocyte cell line derived from a patient showing Bare lymphoma syndrome, and it may be grown attached to plates. Cells were grown in minimum essential medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. BLS-1 cells were cultured in 35-mm diameter collagen-coated dishes for transport studies and in flasks for total RNA extraction. Raji cells were seeded in flasks for all the experiments.

**Transport Measurements**—The specific conditions for uridine uptake in cultured BLS-1 cells were as described previously (31).  $^3\text{H}$ -labeled uridine (Amersham Pharmacia Biotech) was used as a tracer for uptake experiments. In the inhibition studies transport was measured either in the absence or in the presence of several nucleosides at a final concentration of 100  $\mu\text{M}$  (1  $\mu\text{M}$  for NBTI). For  $\text{Na}^+$ -independent transport,  $\text{NaCl}$  in the uptake buffer was replaced by choline chloride. When the incubation time had elapsed the medium was removed and the plates were washed in an ice-cold medium (137 mM  $\text{NaCl}$ , 10 mM HEPES/Tris, pH 7.4). Dishes were drained, and 0.5 ml of Triton X-100 (0.5%) was added. The extract was used for both radioactivity measurement and protein determination (32).

Since Raji cells grow in suspension, the uptake assay differed from that in BLS-1. Essentially, the rapid filtration method previously characterized in our laboratory was used (33, 34). Basically, uptake measurements were started by mixing the cell suspension with the same volume of transport medium, with or without inhibitors, and radionucleosides. To stop the uptake measurements, aliquots of the transport mixture were added to ice-cold Eppendorf tubes containing an upper buffer phase, an intermediate oil layer (dibutylphthalate/bis-(3,5-trimethylhexyl) phthalate (3:2, v/v)) and a lower layer of  $\text{HClO}_4$ /glycerol (1:9, v/v). The tube was immediately centrifuged ( $14,000 \times g$  for 60 s); the supernatants were aspirated, and the radioactivity of the acid extract was measured. The advantages of this technique are described elsewhere (35). D-[1- $^{14}\text{C}$ ]Mannitol (Amersham Pharmacia Biotech) was included in the incubation medium to correct for the extracellular medium trapped in the acid layer. Protein was measured in the transport mixture as described above.

**RNA Isolation, Poly(A) $^+$  RNA Purification, cDNA Synthesis, and PCR Amplification**—Total RNA was extracted from BLS-1 and Raji human B-lymphocyte cell lines and human placenta by the CsCl



**FIG. 1. Major components of nucleoside uptake in human B-lymphocyte cell lines BLS-1 and Raji.** Transport studies were performed as described under "Experimental Procedures." The total uridine uptake is considered 100% and every component is referred to as a portion of the overall incorporation into the cell. *cs*, concentrative uptake sensitive to NBTI; *ci*, concentrative uptake insensitive to NBTI; *es*, equilibrative uptake sensitive to NBTI; *ei*, equilibrative component not inhibitable by NBTI.

method, as described previously (36). Poly(A) $^+$  RNA was purified from placental total RNA using the Poly(A) tract mRNA isolation system (Promega). Then, cDNA was synthesized using the PCR-related reverse transcription system (Promega), and the cDNA/mRNA hybrid was treated with RNase H. Finally, the whole reaction was cleaned up by using the Wizard DNA clean-up system (Promega). The final cDNA solution from placenta was used as template in the PCR reactions. To generate the PCR-generated hENT1 cDNA, 5  $\mu\text{l}$  of the above cDNA reaction was used. The hENT1 oligos hENT5 (5'-GGCCAGGGCCTAG-CAGGCTTCTTT-3', base pairs 713 to 736) and hENT3 (5'-GAGGCTG-GCGAGGTAGCCGTTGGA-3', base pairs 1417 to 1394) derived from the published cDNA sequence (9) were used. The PCR reaction was set up by mixing (final concentration) the following:  $1 \times$  Taq polymerase buffer, 1.5 mM  $\text{MgCl}_2$ , 2.2 mM each dNTP, 0.4  $\mu\text{M}$  each F1/R1 oligo, the cDNA and water to 50  $\mu\text{l}$  of final volume. 50  $\mu\text{l}$  of mineral oil was added onto the reaction. The reaction mix was heated to 94  $^{\circ}\text{C}$  for 5 min and then cooled to 80  $^{\circ}\text{C}$ . Then, 2.5 units of Taq polymerase was added. The PCR conditions were as follows: 1 min, 94  $^{\circ}\text{C}$ ; 2 min, 60  $^{\circ}\text{C}$ ; 3 min, 72  $^{\circ}\text{C}$  for 40 cycles. Finally, the PCR was heated to 72  $^{\circ}\text{C}$  for 10 min and cooled to 4  $^{\circ}\text{C}$  until the samples were run in a 1% agarose gel (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). The fragment was blunted with Klenow fragment and ligated into the *EcoRV* site from BlueScript KS. The hENT1 cDNA sequence was confirmed using the Auto Read sequencing kit and the A.L.F. DNA Sequencer (Amersham Pharmacia Biotech).

**Northern Blot Analysis**—Up to 20  $\mu\text{g}$  of total RNA was fractionated by electrophoresis through a 1% agarose, 3% formaldehyde gel in 20 mM MOPS and 1 mM EDTA, pH 7.4. Application of equal amounts of RNA to each lane was confirmed by the addition of ethidium bromide to the samples before electrophoresis. The gel was treated as described previously (37). RNA was transferred overnight to an Immobilon filter (Amersham Pharmacia Biotech) by capillary action in  $20 \times$  SSC (SSC, 3 M  $\text{NaCl}$ , 300 mM sodium citrate, pH 7.0). RNA was cross-linked to the filter by irradiation with UV light. The filter was prehybridized and hybridized at high stringency following (37). Thus,  $10^6$  cpm/ml of an [ $\alpha$ - $^{32}\text{P}$ ]CTP random primer-labeled hENT1 cDNA was used to hybridize the filters. Filters were washed once for 30 min at 65  $^{\circ}\text{C}$  in  $3 \times$  SSC and 1% lauryl sulfate, once in  $1 \times$  SSC and 1% lauryl sulfate, and once with  $0.2 \times$  SSC and 1% lauryl sulfate before autoradiography. Blots were also hybridized at high stringency, as described above, with a 1-kilobase pair fragment of hTNF- $\alpha$  cDNA and 175 base pairs of a cDNA probe to the rat 18 S ribosomal RNA, used as loading and transfer control.

#### RESULTS

**Characterization of Nucleoside Transport Systems in Human B-lymphocyte Cell Lines**—Nucleoside transport in human B cells was characterized in two cell lines, Raji and BLS-1. Fig. 1 shows the relative contribution of the various transport systems involved in uridine uptake (1  $\mu\text{M}$ ) in both cell lines. BLS-1 and Raji cells showed the same components of transport although their relative activities were markedly different. Raji cells showed higher concentrative uptake than BLS-1 cells. This  $\text{Na}^+$ -dependent transport activity can be separated into two components, an NBTI-sensitive (*cs*) and an NBTI-insensi-

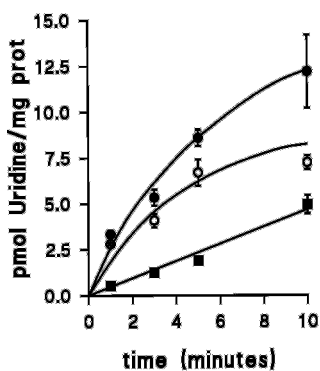


FIG. 2. Time course of uridine transport into BLS-1 cells. Cells were cultured as described under "Experimental Procedures." Human B-lymphocytes were incubated with  $1 \mu\text{M}$  uridine either in a NaCl ( $\bullet$ ) or a choline chloride ( $\circ$ ) medium. Incubation was stopped at the indicated times, and  $\text{Na}^+$ -dependent transport ( $\blacksquare$ ) was calculated by subtracting those rates measured in the choline medium from those measured in the  $\text{Na}^+$  medium. Results are the mean  $\pm$  S.E. of four experiments. *prot*, protein.

tive (*ci*) component. Inhibition using  $100 \text{ nM}$  NBTI instead of  $1 \mu\text{M}$  (Fig. 1) gave similar results. Although the concentrative component of nucleoside transport was lower in BLS-1 cells ( $\sim 25\%$ ), the evidence that *ci* transport was negligible in this cell line (less than 4%) prompted us to use BLS-1 cells to examine this  $\text{Na}^+$ -dependent NBTI-sensitive transport system (see below). The NBTI-resistant fraction of uridine ( $1 \mu\text{M}$ ) uptake may be related to an N1-type transport system, since it was inhibited by  $100 \mu\text{M}$  formycin B (data not shown).

Equilibrative uptake was a major component of nucleoside uptake in BLS-1 cells (about 70% of the total and about 20% of the total in Raji cells) (Fig. 1). Most of this transport activity was NBTI-sensitive, whereas the residual uptake was resistant to uridine and adenosine inhibition at a concentration 100 times higher than that of the substrate. In summary, these data suggest that human B cells show a concentrative, NBTI-sensitive (N5-like or *cs*) transport activity, a concentrative, formycin B-sensitive transport system (N1-like or *cif*), and an equilibrative NBTI-sensitive (*es*) transport activity.

As indicated above, BLS-1 cells were used to characterize this N5-like transport activity.  $\text{Na}^+$ -dependent uridine uptake was linear throughout the 10-min incubation, and thus a 3-min time point was routinely used for kinetic analysis (Fig. 2). Concentration dependence of uridine uptake either in the presence or in the absence of sodium revealed a concentrative component (Fig. 3) with an apparent  $K_m$  of  $11 \pm 3 \mu\text{M}$  and a  $V_{\text{max}}$  of  $28 \pm 4 \text{ pmol uridine/mg protein/3 min}$ . The possible substrate specificity of this concentrative nucleoside transport system was assessed by *cis*-inhibiting  $1 \mu\text{M}$   $\text{Na}^+$ -dependent uridine uptake in the presence of a variety of nucleosides at  $100 \mu\text{M}$  (Fig. 4). Uridine, adenosine, guanosine, and NBTI ( $1 \mu\text{M}$ ) completely inhibited the  $\text{Na}^+$ -dependent fraction of uridine uptake, but cytidine, thymidine, and formycin B did not. These data are consistent with the expression of N5-like activity, as recently described elsewhere (13). To clarify whether NBTI-sensitive equilibrative transport activity was related to the expression of the recently cloned hENT1 transporter, a Northern blot analysis was performed (Fig. 5). hENT1 mRNA was detected in Raji and BLS-1 cell lines and in human placenta, the origin of the first hENT1 cDNA clone.

**Influence of Cell Density on Nucleoside Transport Systems in Human B-lymphocytes**—N1 and N5 transport activities decreased progressively as cell density increased (Fig. 6) and, indeed, were not detected at the highest densities analyzed (Fig. 6b). The equilibrative *es* component was not significantly affected by changes in cell density.

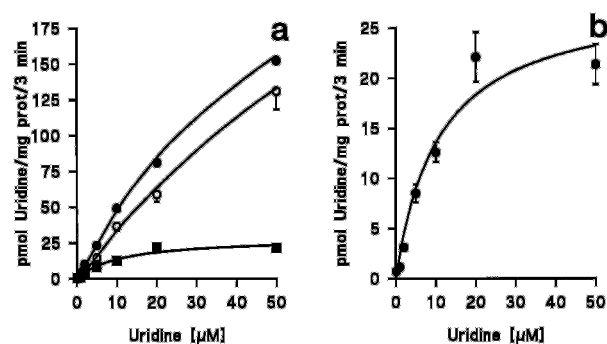


FIG. 3. Concentration dependence of uridine uptake into BLS-1 cells.  $\text{Na}^+$ -dependent uridine uptake was analyzed over a range of different substrate concentrations from  $0.1$  to  $50 \mu\text{M}$ . *a*, total uridine uptake was analyzed in the presence ( $\bullet$ ) or the absence ( $\circ$ ) of NaCl medium as described under "Experimental Procedures." The  $\text{Na}^+$ -dependent uridine uptake ( $\blacksquare$ ) was calculated by subtracting those rates measured in the choline medium from those measured in the  $\text{Na}^+$  medium. *b*, kinetics of the concentrative  $\text{Na}^+$ -dependent uridine uptake. Values are the mean  $\pm$  S.E. of four different determinations. *prot*, protein.

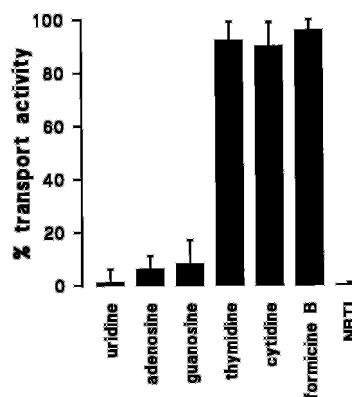


FIG. 4. Effect of different nucleosides and nucleoside analogs on  $\text{Na}^+$ -dependent uridine uptake. Human B-lymphocytes were incubated, as indicated under "Experimental Procedures," in the presence of  $1 \mu\text{M}$  uridine for 3 min, either in the absence or in the presence of selected nucleosides or nucleoside analogs at a concentration of  $100 \mu\text{M}$  ( $1 \mu\text{M}$  NBTI). These compounds were added at the same time as the substrate. Measurements were performed either in a NaCl or choline chloride medium.  $\text{Na}^+$ -dependent uptake was calculated by subtracting the rates measured in choline medium from those obtained in the  $\text{Na}^+$  medium. Results are expressed as percentages of transport activity versus control values (uptake rates measured in the absence of inhibitor). Values are the mean  $\pm$  S.E. of at least five different determinations.

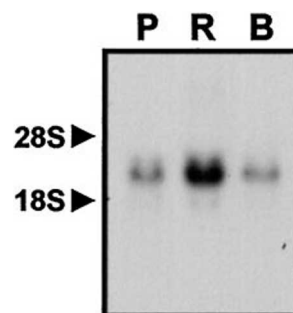


FIG. 5. Northern blot analysis of hENT1 mRNA expression in human placenta and several human B-lymphocyte cell lines. Total RNA was extracted and processed as described under "Experimental Procedures." P, placenta; R, Raji cell line; B, BLS-1 cell line.

**Regulation by PMA and LPS of the Nucleoside Transport Systems in Human B-lymphocytes**—PMA (Calbiochem) and LPS (Sigma) induced a rapid, concentration-dependent effect on N1, N5, and *es* transport activities in Raji cells (Figs. 7 and

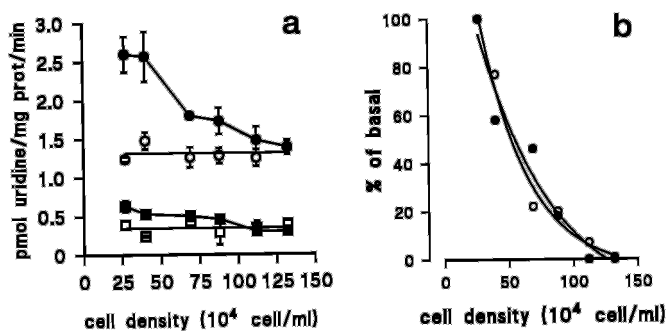


FIG. 6. Influence of cell density on nucleoside transport systems in Raji cell line. Values are the mean  $\pm$  S.E. of three experiments each performed in triplicate. *a*, uridine uptake in the presence ( $\bullet$ ,  $\circ$ ) or in the absence ( $\blacksquare$ ,  $\square$ ) of NaCl. 1  $\mu$ M NBTI ( $\circ$ ,  $\square$ ) was added as described under "Experimental Procedures." Cell density was calculated by counting an aliquot of cell culture. *b*, shows the percentage of the basal uptake ( $27 \times 10^4$  cell/ml) mediated by the concentrative systems in human B cells.  $\bullet$ , N1-like;  $\circ$ , N5-like activities. *prot*, protein.

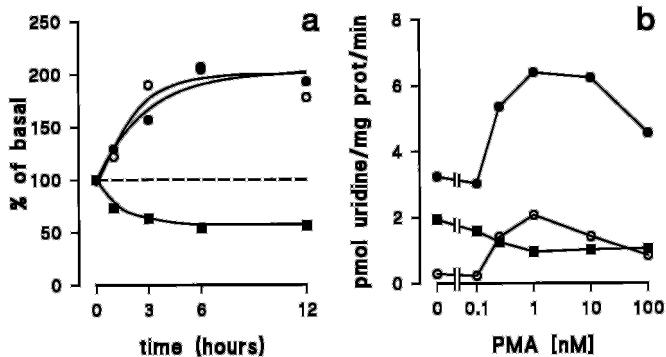


FIG. 7. Effects of PMA on nucleoside transport systems in human B-lymphocytes. Results are the means of at least three independent experiments each done in triplicate. All standard errors are within 15%. *a*, time course of 10 nM PMA effects. Values are the percentage of the basal uridine transport. *b*, dose-dependent effects on uridine uptake after 6 h incubation with 10 nM PMA. Systems N1 ( $\circ$ ), N5 ( $\bullet$ ), and *es* ( $\blacksquare$ ) were monitored as described under "Experimental Procedures." *prot*, protein.

8). The concentrative transport systems were up-regulated and the equilibrative component decreased. These effects appeared to be significant soon after the addition of PMA and LPS, respectively (Figs. 7*a* and 8*a*). As shown in Fig. 7*b* and Fig. 8*b*, the action of these agents was also concentration-dependent.

To determine whether the decrease in *es* transport activity correlates to changes in hENT1 mRNA abundance, Northern analyses were performed in Raji cells grown under the same culture conditions (Figs. 9 and 10). Both PMA and LPS slightly but significantly decreased hENT1 mRNA levels in a time-dependent manner, consistent with the observed changes in transport activity (Fig. 9). After 24 h of incubation with LPS there was a decrease of  $\sim 25\%$  in hENT1 mRNA abundance. This change in the expression was concentration-dependent and of a similar magnitude to that found in nucleoside uptake rates (Fig. 10).

**TNF- $\alpha$  Modulation and Role of PKC in the Regulation of Nucleoside Transport Systems**—PMA and LPS promote TNF- $\alpha$  gene expression and secretion to the medium (26, 28). In this study, treatment of Raji cells with these agents induced accumulation of TNF- $\alpha$  mRNA in a time- and dose-dependent manner (Figs. 9 and 10). To assess whether these effects of PMA and LPS could be mediated by a putative effect of TNF- $\alpha$  on nucleoside uptake, Raji cells were directly incubated in the presence of the cytokine itself. Human recombinant TNF- $\alpha$  (BASH-Knoll, specific activity  $8.1 \times 10^6$  units/mg protein) in-

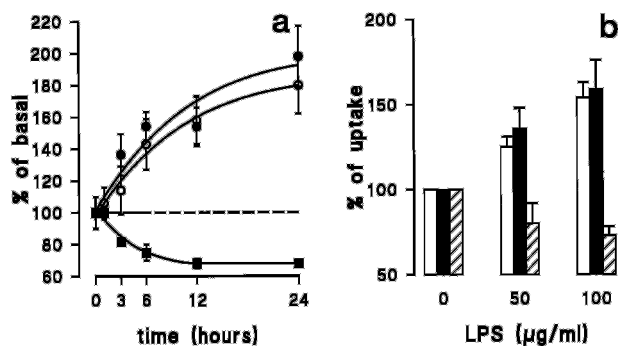


FIG. 8. Effects of LPS on nucleoside transport systems in human B-lymphocytes. Results are the means  $\pm$  S.E. of at least three independent experiments each performed in triplicate. *a*, time course of LPS (100  $\mu$ g/ml) effects. Values are the percentage of the basal uridine transport.  $\circ$ , N1;  $\bullet$ , N5;  $\blacksquare$ , *es*. *b*, dose-dependent effects on uridine uptake after 6 h of incubation. *Open bar* represents N1 system; *closed bar*, N5 system; *hatched bar*, *es* system. Systems were monitored as described under "Experimental Procedures."

duced a decrease in ENT1 mRNA abundance similar to that observed in the presence of PMA or LPS (Fig. 11). Furthermore, TNF- $\alpha$  decreased *es* and increased N1 and N5 transport activities in a manner similar to that observed for PMA and LPS (Fig. 12).

To determine whether the LPS-triggered effect is mediated by protein kinase C activation, we analyzed whether the effects induced by LPS could be blocked when cells were incubated in the presence of bisindolylmaleimide I (BSDM) (Calbiochem), a specific inhibitor of protein kinase C. The addition of BSDM blocked the PMA-, LPS-, and TNF- $\alpha$ -mediated increase in the activity of the concentrative transport systems N1 and N5 (Fig. 12). BSDM equally inhibited the decrease in the activity of the *es* transport system, but only when this was caused by PMA and LPS, not by TNF- $\alpha$  (Fig. 12).

#### DISCUSSION

These results indicate that at least three nucleoside transport systems are present in human B-lymphocytes, an *es* transport system, which appears to be associated with hENT1 expression, and the N1 and N5 concentrative transport systems. Moreover, PMA, LPS, and TNF- $\alpha$  differentially regulate the concentrative and equilibrative transporters, thus suggesting that B cell activation and proliferation are associated with isoform-specific regulation of nucleoside transport systems. This is also the first indication that the recently characterized N5 transport system is not constitutively expressed in B cells and may be highly regulated.

A preliminary report showing NBTI-sensitive Na<sup>+</sup>-dependent nucleoside uptake into freshly isolated leukemia cells (38) did not lead to a more precise kinetic characterization until recently, when Flanagan and Meckling-Gill (13) reported that guanosine transport into NB4 cells, a cell line derived from a patient with acute promyelocytic leukemia, was mostly Na<sup>+</sup>-dependent and inhibited by NBTI. Unfortunately, neither the kinetic properties of this transport system nor its substrate profile have been studied in detail, and the information we have at present may even be misleading. The putative substrate specificity of this N5-type transport system was analyzed by inhibiting nucleoside transport, in a sodium medium only, and with a ratio inhibitor/substrate 10/1, the inhibitor being at a concentration equal to 2-fold the  $K_m$  value for the substrate (13). This approach may have yielded controversial data because it did not take into account the magnitude of the inhibition of the equilibrative transport that measured choline chloride medium. Partial inhibitions triggered by a variety of purine and pyrimidine nucleosides were reported, and they are

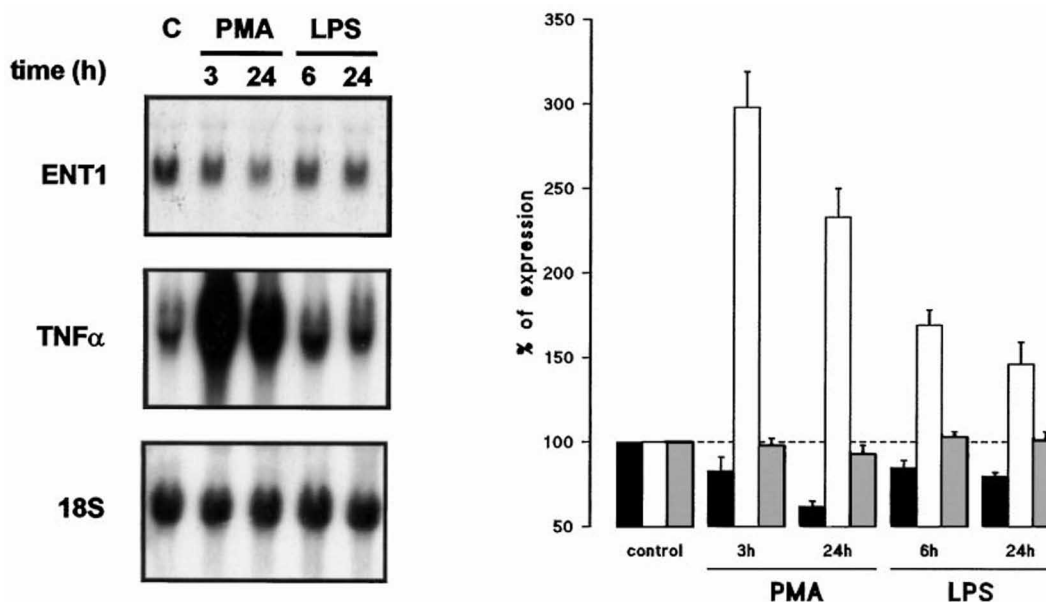


FIG. 9. PMA and LPS effects on mRNA expression of ENT1 and TNF- $\alpha$  in human B-lymphocytes. Left panels, some representative Northern blot experiments are shown. Right panel represents the percentage of the mRNA expression of ENT1 (closed bars), TNF- $\alpha$  (open bars), and 18 S ribosomal band (shaded bars). Values are the means  $\pm$  S.E. of three independent Northern blots each performed with independent samples. PMA (10 nM) and LPS (100  $\mu$ g/ml) were added to cultured B cells, and total RNA was extracted at the desired times and processed as described under "Experimental Procedures."

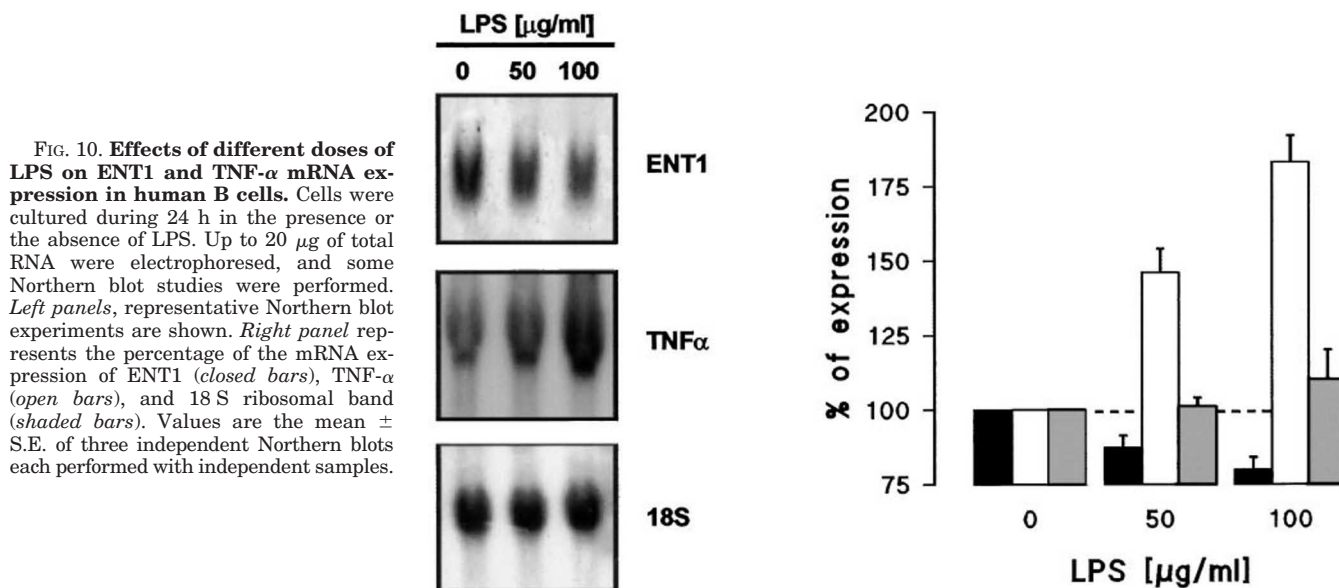


FIG. 10. Effects of different doses of LPS on ENT1 and TNF- $\alpha$  mRNA expression in human B cells. Cells were cultured during 24 h in the presence or the absence of LPS. Up to 20  $\mu$ g of total RNA were electrophoresed, and some Northern blot studies were performed. Left panels, representative Northern blot experiments are shown. Right panel represents the percentage of the mRNA expression of ENT1 (closed bars), TNF- $\alpha$  (open bars), and 18 S ribosomal band (shaded bars). Values are the mean  $\pm$  S.E. of three independent Northern blots each performed with independent samples.

difficult to interpret. In contrast, the inhibition studies performed here using BLS-1 cells, cultured either in Na<sup>+</sup> or in choline chloride media, showed that Na<sup>+</sup>-dependent uridine uptake was completely abolished by purines and NBTI, whereas pyrimidines and formycin B had little effect on this transport activity. The  $K_m$  for uridine of the N5-type transport system, here characterized in BLS-1 cells, was in the low micromolar range, which is similar to other concentrative nucleoside transporters, although it is one-fifth of the  $K_m$  value recently given by Flanagan and Meckling-Gill (13) when using guanosine as a substrate. However, it is now known that the same carrier protein may show this range of  $K_m$  variation for natural nucleosides. hCNT2, when expressed heterologously, takes up inosine with an apparent  $K_m$  of 4.5  $\mu$ M, whereas this kinetic constant reaches 80  $\mu$ M when uridine is used as substrate (16).

The other major component for concentrative nucleoside up-

take in human B cells, N1 or *cif*, has also been detected by others in human acute promyelocytic leukemia NB4 cells (39), murine leukemic L1210 cells (23, 24, 40), and rat macrophages (41), among other cell types. An *es* transport system has also been characterized in a wide variety of lymphocyte-derived cell types (8, 13, 23), which is consistent with recent evidence that hENT-like mRNA species are present in many leukemia cells, such as K562, HL-60, Molt-4 and, as in the present report, the Raji cell line (42).

The coexpression of N1, N5, and *es* transport systems in Raji cells makes it a suitable model with which to analyze the putative effects of cell activation on nucleoside transport activity. Previous data have shown that the differentiation of HL-60 cells induced by Me<sub>2</sub>SO or PMA triggers the activation of a Na<sup>+</sup>-dependent nucleoside transport activity that has not been unequivocally characterized so far (20–22). However, the present report provides the first evidence that both N1 and N5 are

FIG. 11. Effects of human recombinant TNF- $\alpha$  incubation on mRNA expression of hENT1 in human B-lymphocytes. Raji cells were incubated with 1000 units/ml human recombinant TNF- $\alpha$ , and at the desired times total RNA was isolated and processed as described under "Experimental Procedures." Left panel represents a representative Northern blot; right panel shows the means  $\pm$  S.E. of three different blots each performed with independent samples. Closed bar represents ENT1 levels; open bars, 18 S ribosomal band.

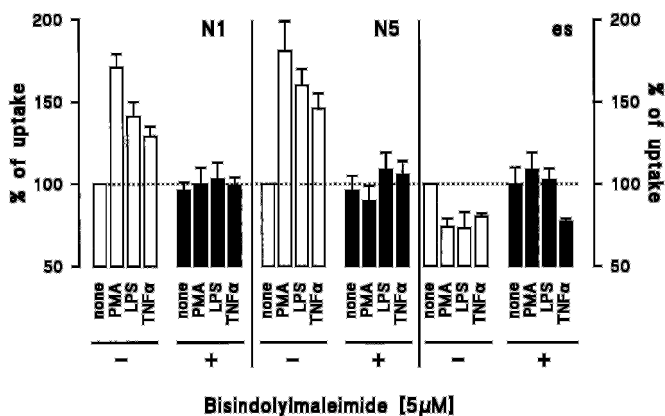
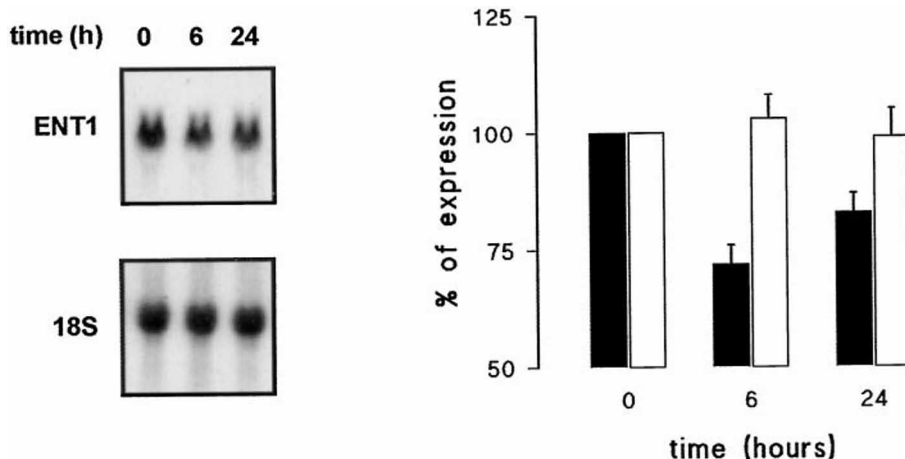


FIG. 12. Specific inhibition of PKC by BSDM on the PMA, LPS, and TNF- $\alpha$  regulation of nucleoside transport systems in human B-lymphocytes. B cells were preincubated for 1 h in the absence (open bars) or in the presence (closed bars) of 5  $\mu$ M BSDM previously to the addition of 10 nM PMA, 100  $\mu$ g/ml LPS, or 1000 units/ml TNF- $\alpha$ . Contribution of different systems to the total uridine uptake was monitored 6 h after the addition of the activators, as described under "Experimental Procedures." Values are the means  $\pm$  S.E. of at least three independent experiments each performed in triplicate.

up-regulated after cell activation triggered by phorbol esters. A pleiotropic effect of these agents on several membrane proteins is possible. Indeed, in purified human chronic leukemic B-lymphocytes, activation by 12-*O*-tetradecanoylphorbol-13-acetate is followed by metabolic labeling of at least 14 unidentified membrane proteins, perhaps including system L for neutral amino acid transport (43). Similarly, T-lymphocyte-related cell lines also show selective up-regulation of membrane transporters following their activation. Indeed, the first amino acid transporters cloned, those belonging to the cationic amino acid transporter family, were isolated by differential hybridization using activated and resting murine T-lymphoma cells (44). This is consistent with the finding that phytohemagglutinin stimulation of T-lymphocytes is followed by increased lysine transport through the  $y^+$  system (the probable cationic amino acid transporters gene product) (45). Since this carrier appears to provide substrates for inducible nitric oxide synthase, the possibility that NO also modulates nucleoside transport in these and other cell types is currently being studied in our laboratory.

The modulation of nucleoside transport by PMA can be understood as a pharmacological effect, but LPS and TNF- $\alpha$ , which play a key role in the physiological activation and proliferation of B cells, are equally able to up-regulate N1 and N5 transport activities. The ultimate physiological role of this response requires further analysis, although a possible relationship between cell growth and up-regulation of concentrative

nucleoside transporters has recently been shown in other cell types (19, 46, 47). These actions appear to be dependent on protein kinase C activation, which is consistent with previous reports based on HL-60 cells induced to differentiate (20–22).

A decrease in equilibrative uridine transport during monocytic differentiation of HL-60 leukemia cells has also been reported by others (22). Indeed, it had been suggested that PMA was able to decrease NBTI-binding sites through a PKC-mediated mechanism that would determine conformational changes of the carriers thus compromising NBTI binding (22). Similar results had been previously reported in other unrelated cell types, such as chromaffin cells (47). Obviously, the recent cloning of *es*- and *ei*-independent carriers demonstrates that NBTI sensitivity cannot be exclusively the result of a mere conformational change induced on a single protein. In this context, our data show that the decrease in NBTI-sensitive equilibrative nucleoside transport is accompanied by a decrease in hENT1 mRNA amounts. Nevertheless, these results do not rule out a first and rapid effect on the transporter itself which is not necessarily dependent on any change in hENT1 mRNA levels, consistent with those observations showing a rapid change in NBTI binding following the activation of protein kinases (22, 47). Again, the evidence that LPS and TNF- $\alpha$  are able to induce similar effects to PMA gives physiological relevance to these observations, although the finding that the TNF- $\alpha$  mediated effect does not require PKC activation raises the question of whether different signal transduction pathways may modulate nucleoside transporter expression in B-lymphocytes.

A variety of nucleoside analogs are currently used in anti-cancer therapy (7). 2-Chlorodeoxyadenosine (cladribine) and fluoroarabinosyladenosine (fludarabine) are putative substrates for the CNT2 gene product (N1 transport system) (15, 48). Little is known about the pharmacological properties of this new N5-type nucleoside transport system. These drugs and others, like gemcitabine, may also be substrates of the *es* transport system encoded by the recently cloned *hENT1* gene (9, 10). Opposite changes in concentrative and equilibrative nucleoside transporters, like those reported here as a consequence of cell activation, are likely to modify intracellular drug bioavailability. Thus, a better knowledge of the regulatory processes involved in nucleoside transport into B cells will be helpful in the elucidation of the factors determining drug targeting and sensitivity in the treatment of hematopoietic and lymphoproliferative malignancies.

**Acknowledgments**—We are grateful to Drs. Francisco J. López-Soriano and Josep M. Argilés (Departament de Bioquímica i Biologia Molecular, Universitat de Barcelona) who kindly donated the pGEM-hTNF plasmid and facilitated the rhTNF- $\alpha$  used in this work. We thank Dr. Janet S. Lee (Memorial Sloan Kettering Cancer Center, New York)

for kindly providing the BLS-1 cell line used in this study. We are grateful to Dr. Neus Potau (Hospital Materno-Infantil de la Vall d'Hebron, Barcelona) who provided the human placenta samples. We also thank Robin Rycroft for editorial help.

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