Photoaffinity labelling of the nucleoside transporter of cultured mouse lymphoma cells

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Nitrobenzylthioniosine (NBMPR), a potent and specific inhibitor of nucleoside transport, is bound reversibly by high affinity sites on nucleoside transporter proteins of erythrocyte membranes and, upon photoactivation, NBMPR molecules become covalently bonded to the sites. This study showed that [^{3}H]NBMPR molecules reversibly bound to intact S49 and L5178Y mouse lymphoma cells became covalently bound upon exposure to UV light. Electrophoretic analysis of plasma membrane fractions from the labelled cells showed that ^{3}H was present in polypeptides which migrated as a major band with an apparent M_r of 45000–65000.

Nucleoside transport *Photoaffinity labelling* *Cultured mouse lymphoma cell* *Nitrobenzylthioinosine* *Nitrobenzylthioinosine covalent binding*

1. INTRODUCTION

The entry of nucleosides into animals cells occurs by a facilitated diffusion process that is specific for nucleosides [1,2]. A number of S^6-substituted 6-thiopurine nucleosides and N^6-substituted adenine nucleosides are potent inhibitors of nucleoside transport [3]; of these, NBMPR is the best studied. NBMPR and congeners bind with high affinity to specific sites on plasma membranes and the occupancy of these sites by NBMPR has been correlated with inhibition of transport activity [5–7]. NBMPR has been used to quantify nucleoside transport elements on a number of cell types [5–7]. In recent studies [8] it was shown that ^{3}H-labelled preparations of the inhibitors of nucleoside transport, N^6-(*p*-azidobenzyl)adenosine and NBMPR, served as photoaffinity reagents that labelled nucleoside transport proteins in erythrocyte membranes. When reversibly bound to the transporter sites, the ^{3}H-labelled ligands became covalently bonded to transporter polypeptides during exposure to UV light, enabling the polypeptides to be identified on SDS polyacrylamide electropherograms. We describe the covalent labelling with [^{3}H]NBMPR of the nucleoside transporter in intact cells from cultured lines of the L5178Y and the S49 mouse lymphomas.

Abbreviations: NBMPR, 6-(4-nitrobenzyl)thio-9-β-D-ribofuranosylpurine (nitrobenzylthioinosine); NBTGR, 2-amino-6-(4-nitrobenzyl)thio-9-β-D-ribofuranosylpurine (nitrobenzylthioguanosine); PBS, Dulbecco's phosphate-buffered saline

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2. EXPERIMENTAL

2.1. *Cell culture*

Stocks of S49, AE_1 and L5178Y mouse lymphoma cells were maintained at 37°C as static cultures in Fischer's medium supplemented with 10% horse serum. To obtain cells for experiments, cultures were expanded to 800 ml in the same medium with antibiotics in roller bottles rotated at 1.5 rpm.

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Cell concentrations were kept below 5×10^6 cells/ml. Cells were harvested by centrifugation ($225 \times g$ for 5 min) and resuspended in serum-free Fischer's medium that lacked bicarbonate and was supplemented with 20 mM Hepes (pH 7.4).

2.2. *Photoaffinity labelling*

Cells (10^6–10^7 cells/ml) were equilibrated at room temperature for 8 min with 1 nM [^{3}H]NBMPR in the absence and presence of 8 μM NBTGR as a competing nonradioactive ligand. This concentration of [^{3}H]NBMPR was sufficient to achieve maximum site-specific binding of NBMPR, defined as the difference between the cell content of [^{3}H]NBMPR acquired in the absence and presence of NBTGR. Incubation mixtures were cooled to 4°C and spun ($225 \times g$ for 5 min) to recover the labelled cells which, after one wash in ice-cold PBS, were resuspended in ice-cold PBS (10^7 cells/ml) for irradiation. The magnitude of reversible site-specific binding of [^{3}H]NBMPR prior to exposure to UV light was determined by centrifuging 0.1-ml portions of the cell suspensions through oil [9] and dissolving the resulting cell pellets in 5% (w/v) Triton X-100 for ^{3}H-counting.

The UV light source was a 200 W mercury arc lamp (Hanovia 654A-0360, Conrad-Hanovia, Newark, NJ) contained in a water-cooled quartz (7874-35, Ace Glass, Vineland, NJ).

Irradiations were carried out at 8°C with stirred cell suspensions contained in quartz cuvettes (10 mm light path) placed about 4 cm from the UV lamp. After specified intervals of exposure to UV light (0–60 sec), samples (0.2–0.4 ml) of cell suspensions were transferred to 5-ml portions of wash medium at 22°C (bicarbonate-free Fischer's medium containing 8 μM NBTGR and 20 mM Hepes at pH 7.4) to displace noncovalently bound ^{3}H-labelled ligand. After 15 min, cells were pelleted ($225 \times g$, 5 min) and the pellets were resuspended in 5-ml portions of fresh wash medium at 22°C. This washing procedure was repeated twice more. The final cell pellets were dissolved in 5% (w/v) Triton X-100 and counted for radioactivity with appropriate quench correction. The assumption was made that cellular ^{3}H content retained after the washing procedure in excess of time-zero values represented covalently bound [^{3}H]NBMPR. Yields of covalent labelling were calculated as percentages of the [^{3}H]NBMPR specifically bound to the transporter sites prior to photoactivation. Control experiments confirmed that site-specific binding of [^{3}H]NBMPR without photoactivation was completely reversible (see, e.g., fig.1A; zero-time point).

2.3. *Membrane preparation and SDS–polyacrylamide gel electrophoresis (PAGE)*

The method of [10] was used to prepare a crude plasma membrane fraction from S49 cells photolabelled with [^{3}H]NBMPR. Cells were disrupted by nitrogen cavitation at 8°C and, after centrifuging the resulting homogenate ($900 \times g$, 5 min, 4°C), the sediment was discarded. The supernatant fraction was spun ($40\,000 \times g$, 20 min, 4°C) to recover a membrane-containing pellet which was dissolved in an SDS-containing buffer [8] with 5 mM dithiothreitol for electrophoresis on SDS–polyacrylamide slab gels using the Laemmli buffer system [11]. Gel lanes were either cut into 2-mm slices for assay of ^{3}H content or were stained with Coomassie blue and scanned at 633 mM with a laser densitometer for comparison with molecular mass standards [12].

2.4. *Materials*

Cell culture materials were purchased from GIBCO, Burlington, Ontario. [G-^{3}H]NBMPR (16 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). NBMPR and NBTGR were prepared in this laboratory [13].

3. RESULTS AND DISCUSSION

Earlier reports showed that in cultured S49 mouse lymphoma cells nucleoside transport was sensitive to NBMPR and that NBMPR binding sites were present on those cells [7,9]. However, the binding sites were absent from AE_1 cells [7], a nucleoside transport-deficient line derived from S49 cells [14]. Nucleoside transport in cultured L5178Y mouse lymphoma cells was also known to be NBMPR-sensitive [9]. The present series of experiments determined whether nucleoside transport sites on intact L5178Y and S49 cells could be photolabelled with [^{3}H]NBMPR. AE_1 cells served as controls for nonspecific labelling.

Fig.1A shows the effects of exposure to UV light of cells with transporter sites occupied by reversibly bound [^{3}H]NBMPR. With both S49 and L5178Y

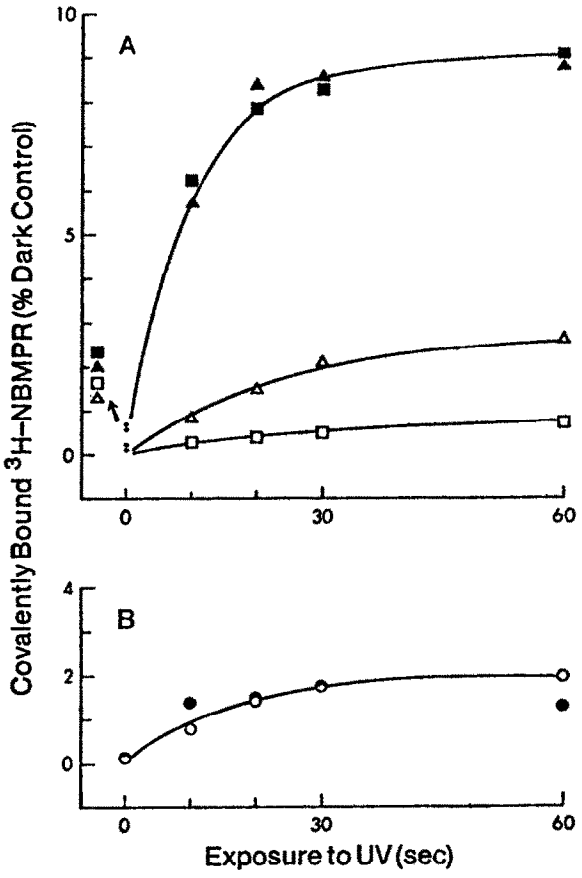

Fig.1. Effect of irradiation time on the covalent attachment of [^{3}H]NBMPR to mouse lymphoma cells. Cells exposed to 1 nM [^{3}H]NBMPR in the absence (closed symbols) or presence (open symbols) of 8 μM nonradioactive NBTGR as described in section 2 were irradiated in standard quartz cuvettes. S49 (▲, △) and L5178Y (■, □) cells were irradiated in the absence of free [^{3}H]NBMPR and AE_1 (●, ○) cells in the presence of free ligand (1 nM). Yields of covalent labelling for S49 and L5178Y cells were calculated as percentages of the site-bound [^{3}H]NBMPR prior to photoactivation (dark control) (1.50×10^{-7} and 1.87×10^{-7} pmol/cell, respectively). AE_1 cells lack high-affinity [^{3}H]NBMPR binding sites. The AE_1 data were therefore calculated as percentages of the corresponding S49 dark control value.

cells, the photolabelling was time-dependent with the maximum retention of ^{3}H observed after 30–60 s of irradiation. Longer irradiation intervals reduced the yield of covalently bound ^{3}H from [^{3}H]NBMPR, as has been reported by others in similar photolabelling experiments with membrane preparations from erythrocytes [8,12]. Covalently bound [^{3}H]NBMPR represented approximately 8% of the specifically bound ligand.

Experiments analogous to those shown in fig.1A were conducted with irradiation medium containing 10 mM dithiothreitol, a free radical scavenger. In the presence of dithiothreitol, the yield of covalent binding was not reduced significantly, suggesting that nonspecific covalent labelling due to migration of free radicals did not occur to the extent observed in previous experiments with erythrocyte membranes [8]. A result consistent with the absence of a dithiothreitol effect was obtained in an experiment (not shown) that measured cellular retention of [^{3}H]NBMPR after photoactivation in the presence of free [^{3}H]NBMPR (1 nM). In this experiment, which was similar to that of fig.1A, site-specific cellular retention of [^{3}H]NBMPR was not increased by the presence of added [^{3}H]NBMPR during photolysis.

The specificity with which photolabelling took place was assessed by comparing [^{3}H]NBMPR photolabelling of S49 cells in the absence and presence of excess nonradioactive NBTGR with that of the transport-deficient AE_1 cells. Both cell types were exposed to UV light in the presence of free [^{3}H]NBMPR (1 nM). In this experiment, the retention of [^{3}H]NBMPR by S49 cells (not shown) was similar to that shown in fig.1A. Fig.1B shows that the [^{3}H]NBMPR retained on AE_1 cells was unchanged in the presence of NBTGR and similar to the low yield obtained for S49 cells in the presence of NBTGR (fig.1A). This result is consistent with the absence of nucleoside transport activity in these cells and their deficiency of NBMPR binding sites [7,14].

The retention of photoactivated [^{3}H]NBMPR by transport-competent S49 cells, the low retention by AE_1 cells, and the properties of the ligand (transport inhibitory, specifically bound) suggest that, in the photoactivation process, nucleoside transport elements were labelled, probably covalently. SDS–PAGE of homogenate fraction containing plasma membranes indicated that [^{3}H]NBMPR retained by the photolabelled cells was covalently attached to cellular material, since polypeptide-associated ^{3}H migrated as a discrete band. Fig.2 shows that in S49 cells polypeptides in the M_r range 45 000–65 000 were labelled by the photoactivation procedure, a result similar to that obtained in the

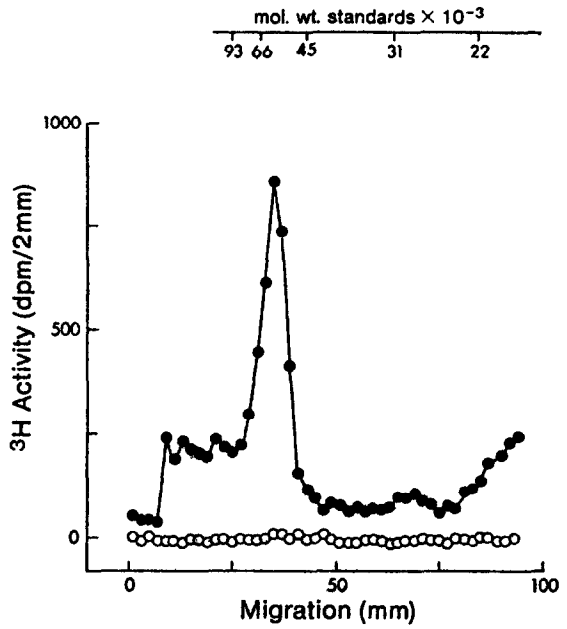

Fig.2. Electrophoretic migration of ^{3}H-bound protein from plasma membranes of S49 cells. Membranes prepared from S49 cells photolabelled with [^{3}H]NBMPR in the absence (●) and prsence (○) of NBTGR were analyzed on an SDS–polyacrylamide slab gel as described in section 2. The membrane-enriched fraction contained 0.22 pmol ^{3}H-labelled ligand covalently bound/mg protein. This compares with a reversible high-affinity [^{3}H]NBMPR binding capacity of 3.6 pmol/mg protein for membranes prepared from control S49 cells not exposed to UV light. The recovery of applied radioactivity from the gel was 86% (250 μg membrane protein loaded per sample).

photoactivation of site-bound [^{3}H]NBMPR on erythrocyte membranes [8,12]. Polypeptides in the M_r range 45 000–65 000 represented 14% of the protein loaded on the gel as judged by densitomer scans of Coomassie blue-stained gels. Additional experiments established that UV exposure did not significantly change the gel electrophoretic profiles of Coomassie blue-stained membrane protein. The radioactive peak shown in fig.2 contained 85% of the protein-associated ^{3}H, corresponding to a covalent labelling of approx. 4% of the high-affinity binding sites present in the membrane preparation; smaller amounts of ^{3}H were found at the gel front and origin. In contrast, no radiolabelling of membrane protein occurred in NBTGR-treated S49 cells.

In conclusion, the data presented here, together with the demonstrated specificity of photoaffinity labelling of the nucleoside transporter of erythrocytes with [^{3}H]NBMPR [12], suggest that the major band of [^{3}H]NBMPR labelling on the S49 cell electropherograms represents nucleoside transporter proteins.

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