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EXTRA-NUCLEAR LOCATION OF HISTONES IN ACTIVATED HUMAN PERIPHERAL BLOOD LYMPHOCYTES AND CULTURED T-CELLS

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Abstract—Dextrin-2-sulphate (D2S) is a sulphated polysaccharide which inhibits human immunodeficiency virus type 1 infection of T-cells by binding to the cell surface. During our investigations of the nature of this interaction, a cell membrane fraction was prepared by ultracentrifugation from the T-cell line, HPB-ALL. Separation of membrane proteins by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and analysis for binding proteins using ligand blotting showed that ^{3}H-D2S bound, in a saturable and displaceable manner, to two regions corresponding to molecular weights of 14,000–18,000 and 28,000–32,000. The *N*-terminal sequences of two of the major protein components in the 14,000–18,000 region were consistent with those of histones H2B and H3. The presence of histone H2B in the cell membrane preparation was confirmed by immunoblotting and enzyme-linked immunosorbent assay using a specific antibody. Histone standards were used to determine the level of each histone in the cell membrane fraction. In addition, the binding of ^{3}H-D2S to purified histone standards was quantified. These results show that all of the binding of ^{3}H-D2S to proteins in the 14,000–18,000 region of the cell membrane preparation can be attributed to the histones present. In contrast to HPB-ALL cells, a cell membrane fraction from freshly isolated human peripheral blood lymphocytes contained very low levels of histones. However, after culture with phytohaemagglutinin for 3 days the cell membrane fraction contained greatly increased levels of histones. To exclude the possibility of contamination of the cell membrane preparation with histones derived from the nucleus, cell membranes were also prepared using an affinity-based method using polyethyleneimine-cellulose. Immunoblotting of adsorbed plasma membranes showed the presence of histone H2B. SDS-polyacrylamide gels stained for protein also indicated that the preparation contained histones H1, H2A, H3 and H4. In further experiments whole cells were used to avoid contamination from nuclear proteins. Lactoperoxidase mediated ^{125}I labelling, a method specific for radiolabelling cell surface proteins, confirmed the presence of histones H2B, H3 and H4 on the surface of HPB-ALL cells. Also, incubation of HPB-ALL cells or phytohaemagglutinin-activated peripheral blood lymphocytes with D2S caused displacement of histones from the cell surface into the supernatant without altering cell viability. In addition, immunocytochemistry of freshly isolated peripheral blood lymphocytes showed that histone H2B was located predominantly in the nucleus. However, in phytohaemagglutinin-activated peripheral blood lymphocytes immunoreactive material was also prominent in the endoplasmic reticulum and on the plasma membrane. These results demonstrate that histones are not confined to a nuclear location.

Key words: histones; cell surface proteins; human immunodeficiency virus; peripheral blood lymphocytes; immunocytochemistry; dextrin-2-sulphate

Cell surface proteins and glycoproteins perform many important functions including recognition, adhesion and receptor-mediated control of cellular events [1–3]. Viruses can bind to cell surface molecules often as the first step in a process which culminates in the entry of the virion into the cell [4–6]. In the case of HIV-1‖ the cell surface determinant CD4 was identified as being important for both the binding of the virion to the cell surface and for its subsequent entry into the cell [7, 8]. However, more recent evidence has shown that the presence of CD4 alone is insufficient to explain the entry of HIV-1 into suspectable cells and that other receptors are also needed [4, 9–11].

D2S is a sulphated polysaccharide which binds to the surface of T-cells and inhibits their infection by a variety of laboratory adapted, cell-free isolates of HIV-1 [12]. Although several sulphated polysaccharides, e.g. dextran sulphate, heparin and fucoidan, have been reported to bind to CD4 [13, 14], the evidence suggests that the anti-HIV-1 activity of D2S does not involve CD4. Firstly, D2S blocks the infection of HeLa cells which lack CD4 as well as HeLa cells transfected with the gene for

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‖ Abbreviations: D2S, dextrin-2-sulphate; HIV, human immunodeficiency virus; PHA, phytohaemagglutinin; PBS, phosphate-buffered saline; PEI, polyethyleneimine; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; AU, absorbance units; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography

human CD4 [15]. Secondly, D2S has no effect on the binding of soluble recombinant CD4 to immobilized gp120 [16]. Thirdly, D2S and soluble recombinant CD4 act in synergy to block the infection of $CD4^+$ T-cells, suggesting that these compounds interfere with different components of the process of infectivity of susceptible cells [15]. Thus, as these results strongly suggest that the anti-HIV-1 activity of D2S is independent of CD4 it is pertinent to ascertain which cell surface compounds bind D2S. Our early results with ligand blots of membrane protein preparations indicated that D2S bound to histones [17]. However, as ligand blotting conditions are quite different from the conditions used to study the binding of D2S to intact cells, it was not possible to conclude that D2S is binding to histones on the cell surface. Similarly, it is necessary to be cautious about linking this observation with the anti-HIV-1 activity of D2S. Nevertheless, the possibility that histones are found on the surface of cells is intriguing, thus, in this paper we have employed a number of techniques to investigate this further using a human T-cell line (HPB-ALL) and activated human peripheral blood lymphocytes, both of which are highly susceptible to infection by HIV-1 and known to bind D2S [18].

MATERIALS AND METHODS

Materials. Size fractionated dextrin was obtained from ML Laboratories plc (Liverpool, UK). All SDS-PAGE reagents were from National Diagnostics (Aylesbury, UK) except for the molecular weight standards which were from Sigma (Poole, UK). Immobilon-P was from Millipore (Watford, UK). [^{3}H]methyliodide and Na^{125}I were obtained from Amersham International (Amersham, UK). Calf thymus histones H2A, H2B, H3, and H4, which have identical sequences to the respective human histones, and calf thymus histone H1, which has extensive sequence similarity to human histone H1, were purchased from Boehringer Mannheim (Lewes, UK). The peptide, PEPAKSAPAPKKGSKKAV-TKAQK was synthesized by Alta Bioscience (Birmingham, UK). Acetonitrile (grade S) was from Rathburn Chemicals (Walkerburn, UK). RPMI 1640 medium, amphotericin B and glutamine were from Flow Laboratories (Irvine, UK). Penicillin and streptomycin were obtained from ICN Laboratories (Oxford, UK). Foetal calf serum was from Imperial (Andover, UK). Ficoll-Paque was from Pharmacia (St. Albans, UK). All electron microscopy reagents were from Agar Scientific Limited (Stansted, UK), except gold labelled goat anti-rabbit IgG antibody which was from Serotec (Kidlington, UK). All other chemicals were purchased from Sigma (Poole, UK) or BDH-Mercke (Lutterworth, UK) and were of AnalaR grade or the best equivalent.

Synthesis of D2S and radiolabelled D2S. The synthesis of D2S from dextrin has been described elsewhere; analysis by infrared spectroscopy and ^{13}C-NMR confirmed that sulphation was in the 2-position [18]. ^{3}H-D2S was prepared by partial alkylation of D2S using [^{3}H]methyl iodide as described previously [18].

Cell preparation. HPB-ALL cells [19] were maintained in RPMI 1640 medium which was supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, 2.5 μg amphotericin B/mL, 250 IU penicillin/mL and 250 μg streptomycin/mL. Human peripheral blood lymphocytes were isolated from heparinized whole blood using Ficoll-Paque, as recommended by the manufacturer. Lymphocytes were activated by culturing them in the presence of 5 μg PHA/mL of supplemented RPMI medium for 3 days.

Membrane preparation by ultracentrifugation. Between 5 and 10×10^7 HPB-ALL cells or $5–10 \times 10^8$ peripheral blood lymphocytes were suspended in 10 mL PBS (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4) and washed three times with PBS by repetitive resuspension and centrifugation at 200 *g* for 5 min. A cell membrane fraction was then prepared by the method described by Blair and MacDermot [20]. Briefly, cells were suspended in 5 mL of ice cold 25 mM Tris-HCl, 290 mM sucrose, pH 7.4 and disrupted by 25 strokes of a Dounce homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min at 4° to remove unbroken cells, nuclei and cell debris and this fraction was retained as a crude nuclear fraction. The supernatant was removed and centrifuged at 105,000 *g* for 20 min at 4°. The resulting pellet was suspended in 5 mL of 25 mM Tris-HCl, 0.25 mM EDTA, pH 7.4 and then centrifuged at 105,000 *g* for 20 min at 4°. The supernatant was discarded and the pellet suspended in 1–2 mL of PBS. Typically this yielded between 1–3 mg of protein, as determined by the method of Lowry *et al.* [21].

Displacement of cell surface histones by D2S. After washing 1×10^7 PHA-activated peripheral blood lymphocytes or 3×10^7 HPB-ALL cells with PBS, as described above, cells were cooled to 4° and suspended in 30 ml PBS containing 0.2 mg/mL D2S or dextrin for 1 hr. The cells were sedimented by centrifugation at 200 *g* for 5 min. The supernatant was centrifuged at 9000 *g* for 20 min to remove any residual cells or cell debris, dialysed three times against 100 volumes of 25 mM ammonium bicarbonate and then lyophilized. The dried residue was dissolved in 250 μL of 25 mM Tris-HCl, pH 6.8 containing 2% (w/v) SDS, 25% (v/v) glycerol, and 0.02% (w/v) bromophenol blue and 20–50 μL were subjected to electrophoresis (see below).

Membrane preparation using polyethyleneimine (PEI) cellulose. PEI cellulose (medium mesh) was prepared as described by Nicotera *et al.* [22]. Between 5 and 10×10^6 HPB-ALL cells were washed twice in PBS and then washed and resuspended in the isotonic buffer, 15 mM sodium acetate, 220 mM sucrose, pH 5.0. The cells were added to 50 mg of PEI cellulose and allowed to mix gently for 1 hr at room temperature. Any free binding sites were blocked by the addition of polyglutamic acid for 1 hr at a final concentration of 1 mg/mL in 15 mM sodium acetate, pH 5.0 containing 220 mM sucrose. The bound cells were then lysed by the addition of ice cold 10 mM Tris-HCl, pH 7.4 for 1 hr on ice. Unbound material was removed by washing three times in the same buffer. Each step of the procedure was followed by light microscopy. Protein bound to

the beads was removed by solubilization in 200 μL of 0.025 M Tris-HCl, pH 6.8 containing 2% (w/v) SDS, 25% (v/v) glycerol, and 0.02% (w/v) bromophenol blue followed by centrifugation for 2 min using a microcentrifuge. The supernatant was subjected to SDS-PAGE.

SDS-PAGE. Cell membrane proteins or individual purified histones were separated using 15% (w/v) acrylamide gels. Protein was applied to the gels in wells 8 mm wide (for staining and immunoblotting) or 35 mm wide (for sequencing and ligand blotting). In some experiments, the acrylamide gels were stained with 0.2% (w/v) Coomassie blue dissolved in 50% (v/v) methanol, 10% (v/v) acetic acid overnight and then destained in several changes of 25% (v/v) methanol, 7.5% (v/v) acetic acid until the background was clear. The intensity of the stained protein bands was quantified using laser densitometry as described below. Alternatively, protein in acrylamide gels was electrotransferred onto nitrocellulose filters in preparation for ligand blotting or immunoblotting, or transferred onto Immobilon-P filters for sequencing.

Ligand blotting. Nitrocellulose filters were stained for protein using 0.1% (w/v) Ponceau red in 1% (v/v) acetic acid for 5 min. The positions of the individual bands were noted and the stain was then removed by incubation in PBS containing 10 mM sodium hydroxide. Filters were blocked for 1 hr using 3% (w/v) BSA dissolved in PBS, washed briefly in PBS and then incubated with 0.1 μM ^{3}H-D2S in 1 mL of PBS for 1 hr at room temperature. Filters were washed twice with 10 mL PBS, cut into 5 mm slices (or specific bands excised) and analysed by scintillation spectroscopy. Non-specific, non-displaceable binding was determined by the addition of 10 μM D2S to the incubation mixture.

Immunoblotting. Immunoblotting was performed using a histone H2B antiserum (prepared as described below) diluted 1:250 in PBS containing 0.1% (w/v) BSA as described previously [23].

Laser densitometry. The intensity of the stained bands was quantified using a LKB Ultroscan XL laser densitometer (Pharmacia Biosystems Ltd, St. Albans, UK) linked to an IBM-XT compatible microcomputer running Gelscan XL 1.21 and Fig.P 6.0 software. The area under the curve for each protein band was expressed as absorbance units × mm (AU mm).

Sequence analysis. After electrotransfer, a strip of the Immobilon-P filter was removed and stained with 0.1% (w/v) amido black dissolved in 10% (v/v) acetic acid, 40% (v/v) methanol for 5 min. The filter was destained by washing three times with 10% (v/v) acetic acid, 40% (v/v) methanol. The *N*-terminal sequences of proteins thus identified was determined according to the method of Matsudaira [24].

Production of anti-histone H2B antibody. The peptide, PEPAKSAPAPKKGSKKAVTKAQK, which corresponds to the *N*-terminus of histone H2B, was dissolved in 0.5 M acetic acid and purified by gel filtration using Sephadex G-25 (2.2 i.d. × 40 cm). The product had the correct amino acid analysis. HPLC was also performed using a Hewlett Packard HP1090A liquid chromatograph (Hewlett-Packard Ltd, Bracknell, UK) with a Nucleosil C_{18} 10 μm column and a gradient of 0–50% (v/v) acetonitrile in the presence of 0.1% (v/v) trifluoroacetic acid over 20 min at a flow rate of 2 mL/min and detection at 210 nm gave a single peak with retention time 6.6 min.

For immunization, the peptide was coupled to a carrier protein using glutaraldehyde. To 2.5 mg purified peptide and 5 mg keyhole limpet haemocyanin dissolved in 2 mL of 0.1 M sodium phosphate buffer, pH 7.2, 100 μL of 2.5% (v/v) glutaraldehyde was added in 20 μL portions over 2 min and allowed to react for 1 hr at room temperature. The mixture was dialysed four times against 500 mL of PBS. New Zealand White rabbits were immunised at intervals of 3 weeks with three injections of 200 μg of the KLH-peptide conjugate mixed with adjuvant. Freund's complete adjuvant was used on the first occasion and thereafter incomplete adjuvant was used. Blood was collected from the marginal ear vein 1 week after the third injection, allowed to clot, centrifuged (950 *g* for 20 min) and the serum collected.

ELISA. This was performed according to Edwards *et al.* [23]. Microtitre plates were coated with either 2 μg/mL of purified histones, 5 μg/mL of peptide, 10 μg/mL of HPB-ALL cell membrane fraction, or 10 μg/mL of HPB-ALL nuclear fraction. Each of these antigens was dissolved in PBS or PBS containing 8 M urea. A series of dilutions between 1:30 and 1:100,000 of antiserum and pre-immune serum were prepared in PBS containing 0.1% (w/v) BSA and added to the coated microtitre plates. The detection of bound IgG using goat anti-rabbit peroxidase was as described previously [23]. Under these conditions the strength of antigen–antibody interactions can be evaluated, however, the assay does not give any indication of the relative levels of antigen in the various preparations.

Radiolabelling of cell surface proteins. Radiolabelling with ^{125}I was performed at room temperature essentially as described by Brenner *et al.* [25] using 7×10^{7} HPB-ALL cells. The cells were >95% viable as determined by trypan blue exclusion. They were washed with PBS (3 × 10 mL) and then resuspended in 1 mL of PBS containing 1 mM magnesium chloride, pH 7.4. To this was added 100 μL of 9 U lactoperoxidase dissolved in PBS containing 1 mM magnesium chloride, pH 7.4 followed by 40 μCi of carrier free Na^{125}I. A total of six additions each of 20 μL of 0.03% (v/v) hydrogen peroxide were then made to the reaction mixture every 3 min. The cells were then washed three times with 10 mL PBS and the membrane fraction prepared by ultracentrifugation as previously described. Proteins in the membrane fraction were separated by SDS-PAGE using 15% gels, stained using Coomassie blue, destained, dried and then subjected to autoradiography.

Immunocytochemistry. Cells were washed three times in PBS and then fixed for 1 hr in 1% (w/v) paraformaldehyde dissolved in 0.1 M sodium phosphate buffer pH 7.2. Fixed cells were dehydrated in a series of solutions containing increasing concentrations of ethanol, from 30% (v/v) to 100% (v/v), 30 min in each solution, and finally in propylene oxide for 5 min. The cells were then pelleted by

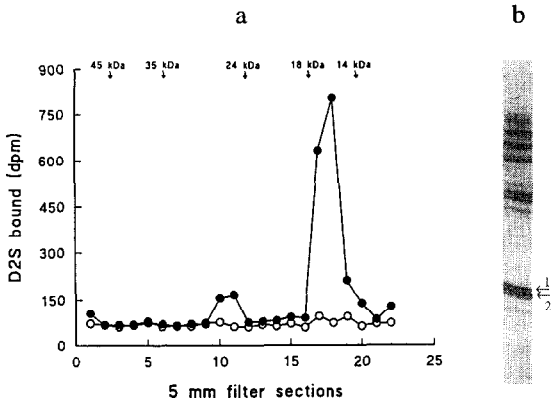

Fig. 1. Ligand blotting and sequencing of D2S binding proteins. (a) Following separation of HPB-ALL cell membrane fraction by SDS-PAGE and electrotransfer onto nitrocellulose the binding of ^{3}H-D2S was determined as described in the Methods section (closed circles). Displaceable binding was determined in the presence of excess unlabelled D2S (open circles). (b) A replicate strip of nitrocellulose stained for protein using amido black. The N-terminal sequences ARTKQTARKSTGGKAPRKQ-LAT (band 1) and PEPAKSAPAPKKGXKKXVTKA- (band 2) were obtained for the protein bands indicated.

centrifugation for 1 min using a microcentrifuge and the supernatant removed. Approximately 1 mL of Araldite I mixture, comprising 20 mL Araldite CY212 Resin, 20 mL dodecenyl succinic anhydride and 125 μL dibutyl phthalate, was added to the cells and mixed for 12 hr at room temperature using a rotatory mixer. The cells were centrifuged as before and the supernatant replaced with 1 mL of Araldite II mixture (20 mL Araldite I and 0.4 mL benzyldimethylamine) and mixed for a further 6 hr at room temperature. Polymerization was performed at 40° for 48 hr in fresh Araldite II mixture. Sections 800–900 nm thick were cut using an Ultracut E (Reichert-Jung) ultramicrotome and collected onto nickel grids. Immunocytochemistry was performed by incubating the sections overnight at 4° with anti-histone H2B antiserum or pre-immune serum diluted 1:100 in 0.1% (w/v) BSA dissolved in PBS. After washing in 0.05 M Tris-HCl, pH 7.4, bound immunoglobulin was detected using 15 nm gold labelled goat anti-rabbit IgG antibody which was diluted 1:20 in 0.05 M Tris-HCl, pH 8.2 containing 1% (w/v) BSA. The sections were incubated for 1 hr at room temperature with this antibody, then washed as before and stained with a methanolic solution of saturated uranyl acetate. Sections were viewed using a Philips-CM10 transmission electron microscope.

Attempts to localise histone H2B using a pre-embedding method were carried out using the method described by Beesley [26].

RESULTS

HPB-ALL cell membrane fraction prepared by ultracentrifugation was analysed by ligand blotting. ^{3}H-D2S bound to two regions corresponding to proteins with molecular weights of 14,000–18,000 and 28,000–32,000 (Fig. 1a). This binding was displaced with a 100-fold excess of unlabelled D2S. Gas phase microsequencing of the proteins in the

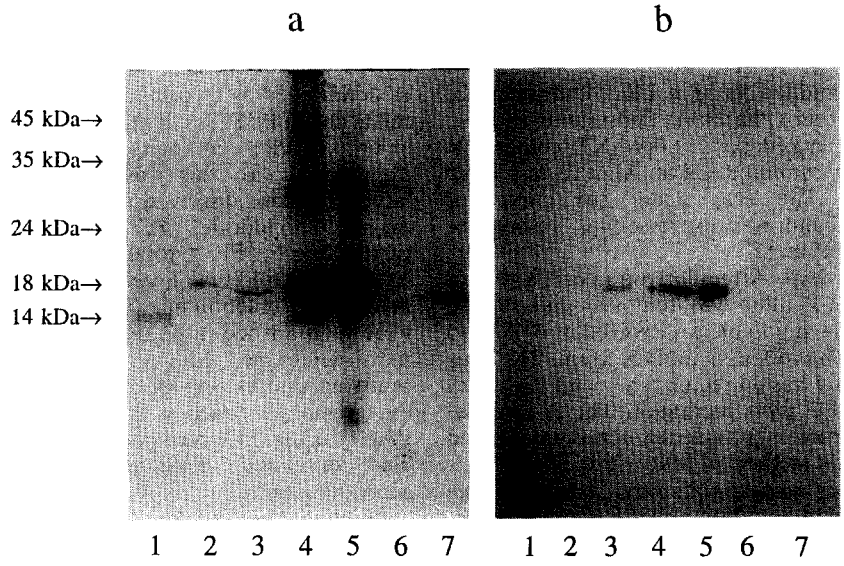

Fig. 2. Comparison of HPB-ALL cell membrane fractions prepared by ultracentrifugation and PEI adsorption. (a) Coomassie blue stained gel following SDS-PAGE and (b) replicate gel analysed by immunoblotting using an anti-histone H2B antibody. To each gel was added 25 μg membrane fraction prepared by ultracentrifugation (lane 4), 40 μg membrane fraction prepared by PEI adsorption (lane 5), 2 μg of purified histones H4 (lane 1), H3 (lane 2), H2B (lane 3), H1 (lane 6) and H2A (lane 7).

14,000–18,000 region was attempted (Fig. 1b). The N-terminal sequences of two of these proteins were found to be PEPAKSAPAPKKGXKKXVTKA and ARTKQTARKSTGGKAPRKQLAT. These sequences are consistent with the N-termini of the histones H2B and H3, respectively. The HPB-ALL cell membrane preparation was analysed by SDS-PAGE. This showed the presence of bands corresponding in electrophoretic migration to purified histones H2B, H2A, H3 and H4 (Fig. 2a). An antibody against histone H2B bound specifically to purified histone H2B and to a single band in the HPB-ALL cell membrane preparation (Fig. 2b).

Purification of membrane proteins from HPB-ALL cells was also undertaken using PEI-cellulose. Analysis of the resulting membrane preparation by SDS-PAGE showed that histones were present as determined by their electrophoretic mobility compared with purified histones and by immunoblotting using an anti-histone H2B antibody (Fig. 2). This procedure relies on the effectiveness of the blocking step in preventing intracellular histone from binding to the PEI-cellulose. Experiments in which PEI-cellulose was first blocked with polyglutamic acid prior to addition of whole cells, membrane or nuclear fraction failed to bind any protein. Also, following binding of cells and then blocking, addition of lysed nuclear fraction had no effect on the level of histones in the resultant membrane fractions. Thus, it is concluded that the blocking procedure was effective and that contamination by intracellular material was minimal.

The binding of ^{3}H-D2S to histones was measured in ligand blotting experiments with each of the purified histones H1, H2A, H2B, H3 and H4. Over the range of 2–10 μg of histone there was a linear relationship between the binding of ^{3}H-D2S and the amount of each histone loaded onto the gel. Quantitatively, the binding of ^{3}H-D2S to the histones was in the order H1 > H2A > H2B > H3 > H4 (Table 1).

In the cell membrane preparation, histones H2A, H2B, H3 and H4 were clearly separated from each other and from other membrane proteins (Fig. 2). Quantification of the individual bands was by comparison with purified standards (Table 2). On the basis of the level of each histone and the amount of ^{3}H-D2S which they bind, it was calculated that in the HPB-ALL cell membrane preparation, the theoretical ^{3}H-D2S binding to H2A, H2B, H3 and H4 is 0.17, 0.13, 0.08, and 0.13 μg ^{3}H-D2S/mg membrane protein, respectively; i.e. a total of 0.51 μg ^{3}H-D2S/mg membrane protein. The actual binding of ^{3}H-D2S to the 14,000–18,000 region of the cell membrane preparation was very similar, 0.50 ± 0.04 μg D2S/mg membrane protein (r = 0.997, N = 5) when measured using between 25 and 150 μg of membrane protein. Thus, the binding of ^{3}H-D2S to the 14,000–18,000 region in the HPB-ALL cell membrane preparation can be accounted for by binding to histones H2A, H2B, H3 and H4.

Table 1. Binding of ^{3}H-D2S to purified histones

Histone	^{3}H-D2S binding (μg/mg protein)
H1	15.4 ± 0.5
H2A	12.2 ± 0.5
H2B	9.0 ± 0.7
H3	4.1 ± 0.2
H4	3.1 ± 0.2

Each of the histones was subjected, over a range of concentrations, to ligand blotting as described in the Methods section. Each value is the mean ± SEM of 10 determinations. The relationship between ^{3}H-D2S bound and histone loaded was linear with regression coefficients >0.98 in all cases.

Table 2. Quantification of histone present in HPB-ALL cell membrane preparation

Histone	Histone concentration (μg/mg membrane protein)
H2A	13.6 ± 0.6
H2B	14.3 ± 0.5
H3	19.0 ± 0.8
H4	41.0 ± 2.9

Samples containing between 15 and 35 μg of HPB-ALL membrane preparation was subjected to SDS-PAGE and stained with Coomassie blue. The level of each histone was determined using laser densitometry by comparison with purified standards. For each histone, in the range of 0.25–1.50 μg, there was a linear relationship between the absorbance measured and the amount of histone added; this was 0.39 ± 0.01 (r = 0.998; N = 10), 0.33 ± 0.01 (r = 0.998; N = 10), 0.21 ± 0.01 (r = 0.971; N = 10), and 0.10 ± 0.01 AU, mm/μg histone (r = 0.985; N = 10) for histones H2A, H2B, H3 and H4, respectively. Each value shown is the mean ± SEM of six determinations.

The level of histone H2B in the HPB-ALL cell membrane fraction was also measured by quantitative immunoblotting using an anti-histone H2B antibody. There was a linear relationship between band intensity and the amount of purified histone H2B used (0.49 ± 0.01 AU mm/μg histone H2B, r = 0.991, N = 10). Quantitative binding of the antibody to the membrane preparation was established as 6.3 ± 0.1 AU mm/mg membrane protein (r = 0.979, N = 10). Thus, it was calculated that the preparation contained 12.9 μg histone H2B/mg of membrane protein, a figure which is comparable to the value obtained using the Coomassie blue staining method (14.3 ± 0.5 μg histone/mg of membrane protein).

In ELISA, the anti-histone H2B antibody bound strongly to the immunising peptide (Fig. 3c) and to purified histone H2B (Fig. 3d), whether these antigens were added in PBS or PBS containing 8 M urea (Fig 3c and d). When the HPB-ALL cell membrane fraction and the nuclear fraction were coated onto microtitre plates in PBS the antibody bound poorly (Figs 3a and b). In contrast, when these fractions were first dissolved in 8 M urea (to denature and dissociate proteins) and then coated onto microtitre plates, the antibody bound strongly to both (Figs 3a and b). Thus, the epitope for the antibody is occluded in intact membrane and nuclear

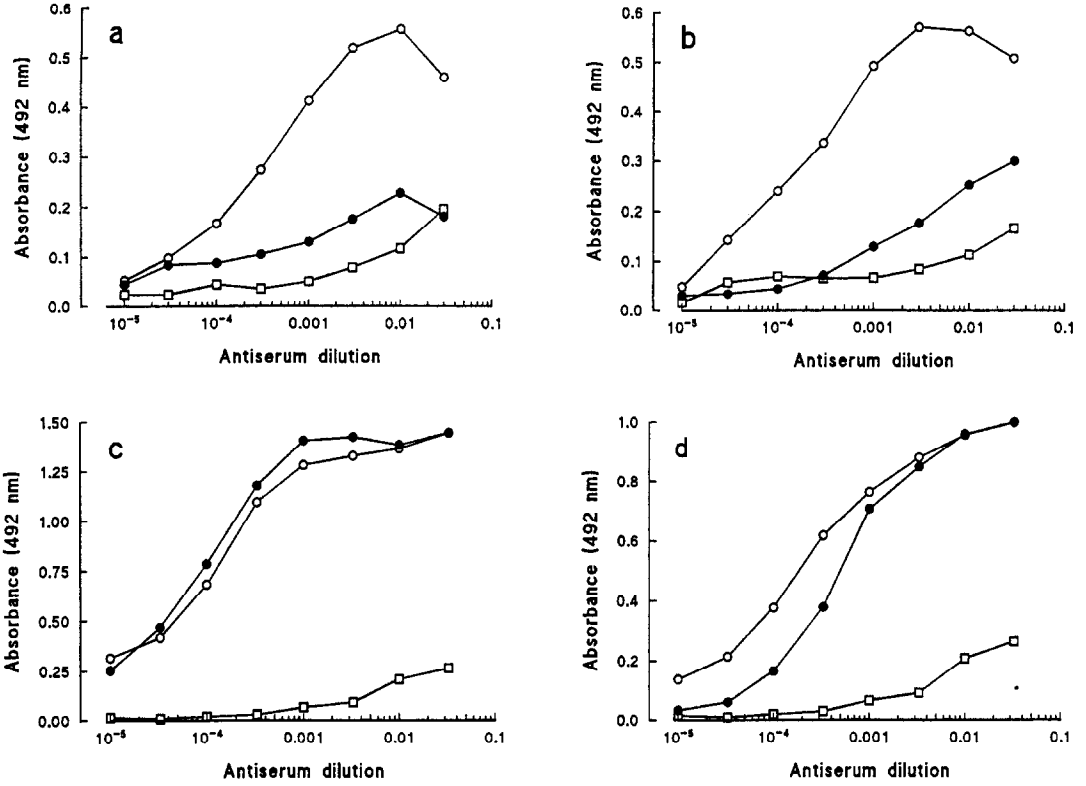

Fig. 3. Relative binding of anti-histone H2B antibody to HPB-ALL membrane fraction. Microtitre plates were coated with (a) HPB-ALL membrane preparation, (b) HPB-ALL cell crude nuclear fraction, (c) peptide PEPAKSAPAPKKGSKKAVTKAQK, or (d) purified histone H2B, dissolved/suspended in PBS (closed circles) or PBS containing 8M urea (open circles). A series of dilutions of antiserum raised against the peptide PEPAKSAPAPKKGSKKAVTKAQK coupled to haemocyanin (circles) or pre-immune serum (squares) was added and antibody binding was determined as described in the Methods section. The binding of pre-immune serum was similar whether antigens were dissolved in PBS or PBS containing 8M urea. For simplicity, binding to antigens dissolved in PBS is shown. Each point is the mean of two determinations and the data shown are from a typical experiment representative of three experiments with similar results. The binding of the antibody to purified histones H1, H2A, H3 and H4 was negligible when examined under these conditions.

fractions, indicating that histone H2B is complexed with other proteins. In the nuclear fraction it is likely that the epitope is masked through complexing with other histones. Binding of immunoglobulin from pre-immune serum to peptide, purified histone H2B, membrane and nuclear fractions, dissolved in PBS or PBS containing 8 M urea, was negligible (Fig. 3).

Cell membrane fractions of human peripheral blood lymphocytes were prepared using the ultracentrifugation method. Freshly isolated lymphocytes yielded 0.9 mg protein from 6×10^8 cells. In a second preparation, an equal number of lymphocytes were first cultured for 3 days in the presence of PHA. This yielded 1.9 mg protein from a total of 9×10^8 cells. SDS-PAGE of the membrane fractions showed a striking increase in the level of histones in activated cells compared with fresh cells, irrespective of whether the analysis is based on cell number or protein concentration (Fig. 4a). Immunoblotting of the same fractions also showed a large increase in the level of histone H2B (Fig. 4b). Quantification showed an increase from 6.2 to 31.9 μg histone H2B/mg membrane protein, or, in terms of cell number, from 0.9 to 6.7 μg histone H2B/10^8 cells.

Labelling of the cell surface proteins on HPB-ALL cells was performed using ^{125}I and lactoperoxidase. The cell membrane fraction was prepared by ultracentrifugation and 15 μg of protein was subjected to SDS-PAGE. Following autoradiography of the gel, three bands were detected which correspond to the migration of histones H3, H2B and H4 (Fig. 5a). Histones H2A and H1 were not radiolabelled. However, histones H1, H2A, H2B, H3 and H4 were present when the gel was stained for protein (Fig. 5b). The protein bands in the 14,000–18,000 region were excised from the polyacrylamide gel and γ-radioactivity measured. The results were 384, 401 and 1507 dpm for the bands corresponding to histones H3, H2B and H4, respectively. The background measured from an adjacent gel slice was 72 dpm.

After incubation of HPB-ALL cells with 0.2 mg/mL D2S or dextrin at 4°, cell viability, as determined

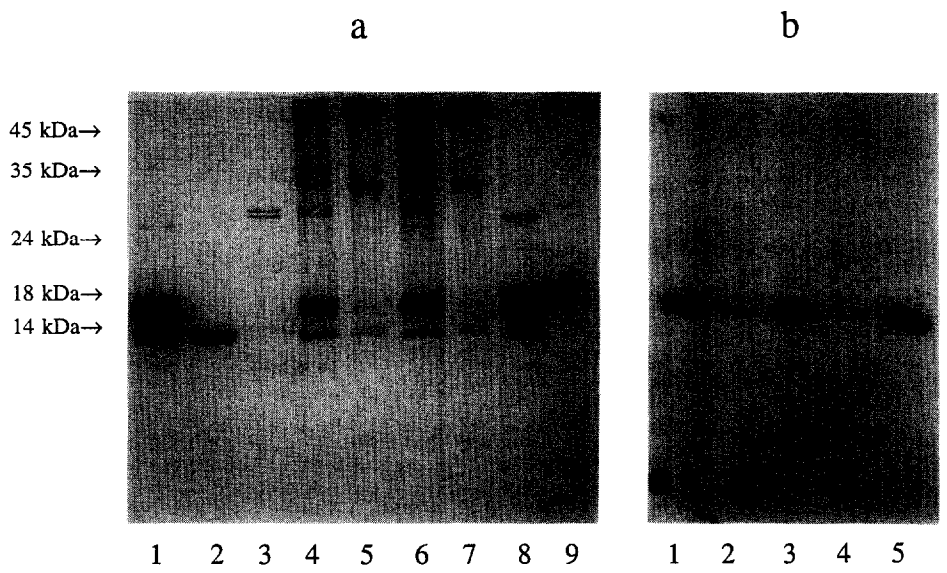

Fig. 4. Expression of histones in the membrane fraction of unactivated and activated lymphocytes. (a) Coomassie blue stained gel following SDS-PAGE of 5 μg histone H2A (lane 1), 5 μg histone H4 (lane 2), 2 μg histone H1 (lane 3), 5 μg histone H2B (lane 8), 3 μg histone H3 (lane 9), 20 μg PHA-activated peripheral blood lymphocyte membrane preparation (lanes 4 and 6), and 20 μg and 16 μg unactivated peripheral blood lymphocyte membrane preparation (lanes 5 and 7, respectively). Lanes 4 and 5 contain an equal amount of protein and lanes 6 and 7 material from an equal number of peripheral blood lymphocytes (8×10^6). (b) Immunoblot developed using an anti-histone H2B antibody. To the gel was applied 5 μg PHA-activated peripheral blood lymphocyte membrane preparation (lanes 1 and 3), 5 μg and 4 μg of unactivated peripheral blood lymphocyte membrane preparation (lanes 2 and 4, respectively), and 0.5 μg purified histone H2B (lanes 5). Lanes 1 and 2 contain an equal amount of protein and lanes 3 and 4 material from an equal number of peripheral blood lymphocytes.

by trypan blue exclusion, was >95%. However, treatment of the cells with D2S, but not dextrin, caused histones H2A, H2B, H3 and H4 to appear in the incubation mixture supernatants, as determined by Coomassie blue staining of proteins and by immunoblotting of histone H2B following SDS-PAGE (Fig. 6). Histones were also displaced from PHA-activated human peripheral blood lymphocytes (Fig. 6). Similar results were found if cells were incubated with D2S at 37°. No displacement of histones was found if the concentration of D2S was reduced by 10-fold.

Immunocytochemistry of freshly isolated peripheral blood lymphocytes using an anti-histone H2B antibody and analysis by electron microscopy showed that histone H2B was located predominantly in the nucleus, with some immunoreactivity detected in the endoplasmic reticulum and on the plasma membrane (Fig. 7a). After activation of peripheral blood lymphocytes with PHA there was an increase of immunoreactivity in the plasma membrane, the endoplasmic reticulum and the nucleus (Fig. 7b). In both activated and unactivated cells some elements of cytomembranes were clearly visible, even though no specific techniques were employed to reveal them: extensive tissue processing for optimal morphology, and retention of antigenicity being mutually exclusive. No immunostaining was found in controls where antiserum was replaced with pre-immune serum, antiserum was omitted, or where the gold-labelled detecting antibody was omitted. Attempts to perform immunocytochemistry using a pre-embedding technique were unsuccessful. This is consistent with the properties of the antibody described above.

DISCUSSION

The results of this study indicate that histones are not confined to a nuclear location in activated human peripheral blood lymphocytes or cultured T-cells. Using an HPB-ALL cell membrane fraction which was prepared by ultracentrifugation, it was shown that under ligand blotting conditions ^{3}H-D2S binds specifically to two regions with molecular weights of 14,000–18,000 and 28,000–32,000. Histones H2B and H3 in the 14,000–18,000 region were identified by *N*-terminal sequencing and the presence of histone H2B in the membrane preparation was confirmed by immunoblotting and ELISA using a specific antibody.

The initial step in the preparation of the cell membrane fraction by the ultracentrifugation method requires cell disruption using a Dounce homogenizer. As it was possible that histones detected in this preparation may originate from the nucleus, an alternative method of preparing cell membranes was also used. The method, using PEI-cellulose, relies upon the interaction of negatively charged cell membrane with positively charged PEI. This

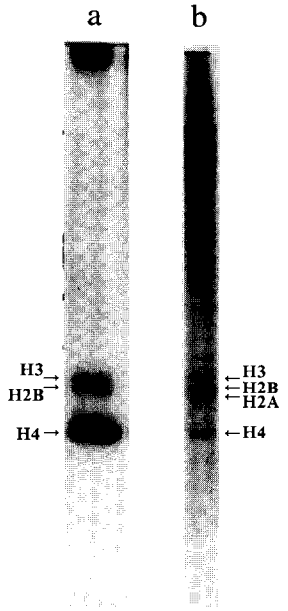

Fig. 5. Radiolabelling of HPB-ALL cell surface proteins. Intact HPB-ALL cells were radiolabelled with ^{125}I using lactoperoxidase. The cells were then homogenised and a membrane fraction prepared by ultracentrifugation and separated by SDS-PAGE. (a) Exposure of the gel to X-ray film for 2 days. This exposure shows that histones were preferentially labelled. Longer exposures show that most membrane proteins were radiolabelled albeit less markedly. (b) The same gel stained with Coomassie blue. The positions of histones are indicated.

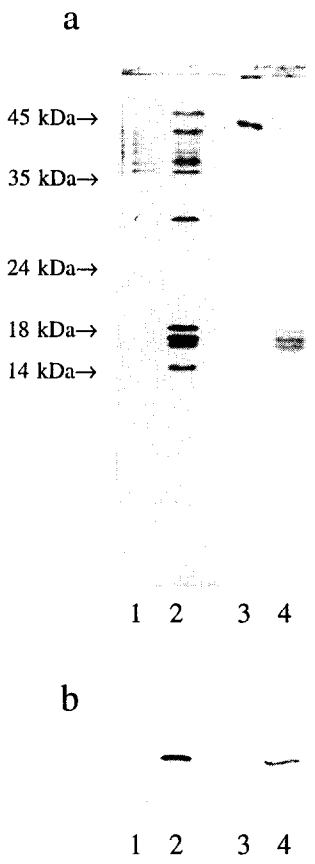

Fig. 6. Displacement of cell surface histones by incubation with D2S. HPB-ALL cells and PHA-activated human peripheral blood lymphocytes were incubated with 0.2 mg/mL D2S or dextrin, the supernatants were collected, dried and dissolved in 250 μL electrophoresis buffer as described in the text. (a) Coomassie blue stained gel of 20 μL HPB-ALL cell supernatant preparation following incubation of cells with dextrin (lane 1) or D2S (lane 2), and 100 μL of PHA-activated human peripheral blood lymphocyte supernatant preparation following incubation with dextrin (lane 3) or D2S (lane 4). (b) Immunoblot of the same samples developed using an anti-histone H2B antibody. The volumes of the samples applied to the gel were half of those used for Coomassie blue staining. Immunoreactivity in the 14,000–18,000 region corresponding to the migration position of histone H2B is shown.

procedure was devised for preparing a highly purified plasma membrane fraction from hepatocytes [22]. As whole cells are bound to PEI-cellulose and free binding sites on PEI-cellulose blocked, prior to cell lysis, the possibility of contamination by nuclear proteins is considerably reduced. Nevertheless, the resulting membrane fraction, when subjected to SDS-PAGE and staining with Coomassie blue, was found to contain histones. The presence of histone H2B was confirmed by immunoblotting using a specific antibody. Thus, these results are in agreement with those obtained using a membrane fraction prepared by ultracentrifugation.

The cell surface location of histones was also studied using human peripheral blood lymphocytes. Cell membrane fractions were prepared using the ultracentrifugation method as used to prepare HPB-ALL cell membrane fraction. However, unlike the HPB-ALL cell membrane fraction, the cell membrane fraction of freshly isolated peripheral blood lymphocytes contained very low levels of histones, as determined by SDS-PAGE and by immunodetection of histone H2B. In contrast, after culture with PHA, the cell membrane fraction of peripheral blood lymphocytes from the same donors was found to contain greatly increased levels of histones.

In ligand-blot studies ^{3}H-D2S was shown to bind to purified histones H1, H2A, H2B, H3 and H4. There was a linear relationship between ^{3}H-D2S binding and the amount of each histone loaded with binding in the order H1 > H2A > H2B > H3 > H4. In addition, the amount of each of the histones H2A, H2B, H3 and H4 in the 14,000–18,000 region of the HPB-ALL cell membrane preparation was determined using laser densitometry. The binding of ^{3}H-D2S to the histones in the HPB-ALL cell membrane preparation was compared with the binding of ^{3}H-D2S to known amounts of purified histones. This showed that the binding of ^{3}H-D2S to the 14,000–18,000 region of the HPB-ALL cell membrane preparation could be accounted for entirely by its binding to histones H2A, H2B, H3

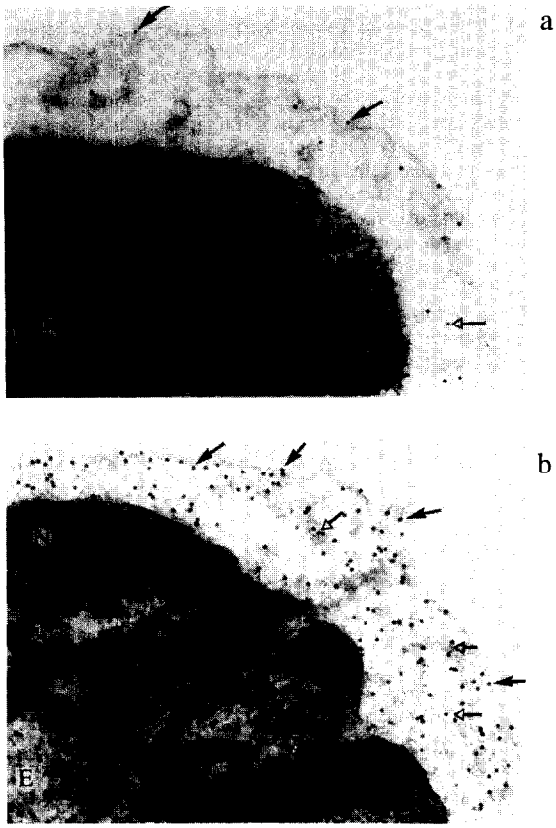

Fig. 7. Immunocytochemistry of histone H2B in lymphocytes. (a) Electron micrograph of a portion of an unactivated peripheral blood lymphocyte immunostained with anti-histone H2B antibody and visualised using an immunogold conjugate. Note that gold labelling is predominantly on the nucleus, N, while some immunoreactivity is present on the plasma membrane (solid arrowheads) and endoplasmic reticulum (open arrowhead). (b) Electron micrograph of a portion of a PHA-activated lymphocyte immunostained with anti-histone H2B antibody and visualised using an immunogold conjugate. Note that there is elevated immunoreactivity in the nucleus, N, and on the plasma membrane (solid arrowheads). There is extensive immunoreactivity on the endoplasmic reticulum (open arrowheads), which would be wholly consistent with elevated *de novo* histone synthesis in the cytoplasm. Decondensed euchromatin, E, is typical in activated cells.

and H4. Thus, the possibility that D2S may be binding to other protein(s) migrating to the 14,000–18,000 region is remote.

There was also some binding of ^{3}H-D2S to a protein band in the region of 28,000–32,000. This corresponds to the migration position of histone H1 on SDS-PAGE. However, as it was not possible to distinguish a band corresponding to histone H1 from the surrounding protein bands, the amount of histone H1 in the membrane preparation could not be quantified by the Coomassie blue/laser densitometry technique and, consequently, a specific band could not be identified for sequencing studies. Therefore, it was not possible to determine whether the binding of ^{3}H-D2S to the 28,000–32,000 region was due to the presence of histone H1.

As it was possible that the histones found in the membrane preparations were due to nuclear contamination, further experiments were performed using whole cells. Radiolabelling of cell surface proteins was performed using ^{125}I. Following electrophoretic separation, radioactively labelled bands were found which correspond in their electrophoretic mobility to the position of purified histones H2B, H3 and H4. These bands also coincide with the position of ^{3}H-D2S binding and immunoreactivity with the anti-histone H2B antibody. However, neither histone H2A nor histone H1 were radiolabelled with ^{125}I. Iodination of purified chromatin gives a similar pattern of labelling [27] and it was suggested by these authors that tyrosine residues in histone H2A but not histone H2B are occluded due to the physical interaction of these two histones. In the present study, the antibody raised against the N-terminus of histone H2B bound strongly to purified histone H2B and to denatured membrane protein, but not native membrane protein. These observations suggest that histones in the plasma membrane may be present as a complex.

Further evidence for the location of histones on the surface of activated human peripheral blood lymphocytes and cultured T-cells was obtained by incubation of intact cells with D2S. This treatment had no effect on cell viability, nor was there any visible change in the integrity of the cells. However, treatment with D2S caused histones to appear in the supernatant. As cells remained intact is it most likely that the histones were displaced from the cell surface.

As the anti-histone H2B antibody was unable to bind to histone H2B in the cell membrane fraction under native conditions, it was not possible to study the distribution of this protein on the surface of living cells. However, the antibody did bind to histone H2B in whole cells which had been fixed and processed for examination by electron microscopy. It appears that the embedding and cutting process reveals the epitope for the antibody as has been found previously [28]. Post-embedding techniques at the electron microscope level are particularly appropriate for this kind of investigation as there is no possibility of antigen lability in fixed tissue [29]. Examination under electron microscopy showed that in freshly isolated peripheral blood lymphocytes, histone H2B was located predominantly in the nucleus. However, after activation of lymphocytes using PHA, there was a general increase in the amount of immunoreactive material not only in the nucleus but also in the cytosol and plasma membrane. This is consistent with the results of SDS-PAGE and immunoblotting analyses of the cell membrane fraction, although quantitative assessment of immunogold grains was not undertaken due to the potential for variation in residual antigenicity as a result of the presence of different microenvironments in electron microscope blocks [30]. The high levels in the cytosol might be explained by an increase in the synthesis of histone H2B, but this has yet to be investigated.

The origin of extra-nuclear histone and its function outside the nucleus is not known. However, the

location of nuclear material on the surface of cells has been reported in a number of previous studies and is thought to be involved in the pathogenesis of systemic lupus erythematosus. These reports have described the presence of nuclear material on the surface of a variety of different cell types including lymphocytes [31, 32], Raji cells [33], cytotoxic T-cells [34], endothelial cells [35], monocytes [36], and mouse B-cells [37].

Sulphated polysaccharides have been found to bind to proteins present in cell membrane preparations which have molecular weights under SDS-PAGE conditions that are similar to histones. It has been shown that [^{3}H]heparin binds to cell membrane preparations from the human lung carcinoma cell line LX-1 [38]. The N-terminal sequences of two of the binding proteins were determined and shown to be identical to histones H2A and H2B, as found here. McClure *et al.* [16] have also shown that ^{125}I-fucoidan binds to cell membrane proteins in the range of 14,000–20,000 prepared from the human T-cell line C8166. Although neither the identity nor the importance of proteins in this region were determined, this result is consistent with fucoidan binding to histones.

In conclusion, we have shown that histones are readily detectable on the surface of HPB-ALL cells and PHA-activated human peripheral blood lymphocytes, but not on freshly isolated lymphocytes. D2S has been shown to bind in a specific, displaceable and saturable manner to whole cells [18]. In addition, D2S blocks infection of HPB-ALL and PHA-activated peripheral blood lymphocytes by HIV-1 [18, 39], whereas unactivated peripheral blood lymphocytes cannot be infected by HIV-1 [39]. We have been unable to link directly the presence of histones on the cell surface with the susceptibility of cells to infection by HIV-1 and the ability of D2S to block the entry of HIV-1 into these cells.

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