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Detection of soluble class I molecules (non HLA-A or HLA-B) in serum, spleen membranes and lymphocytes in culture*

Soluble major histocompatibility complex class I molecules (sHLA) present in human serum can be resolved by gel filtration into two different peaks with an apparent molecular mass of about 200 kDa (30% of the total) and 50–60 kDa (60%–70%). The serological analysis of the peaks shows that A or B specificities can only be detected in the 200 kDa peak while both are recognized by the monomorphic W6/32 monoclonal antibody (mAb) and anti-β_2-microglobulin mAb. Such sHLA (non HLA-A or -B) molecules are released from human spleen membranes upon incubation at 37 °C and have been purified by affinity chromatography with mAb W6/32 bound to Sepharose. The molecular mass analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the sHLA (non HLA-A or -B) and of the classical HLA-A or -B antigens still bound to the membranes and purified from the same membranes after detergent solubilization does not show a significant difference, indicating that sHLA do not represent proteolytic fragments of the classical HLA-A or -B antigens. The presence of sHLA (non HLA-A or -B) has also been detected in the supernatants of lymphocyte cultures and increases dramatically upon stimulation by mitogens. The effect of pokeweed mitogen, phytohemagglutinin, *Staphylococcus aureus* Cowan strain and phorbol 12-myristate 13-acetate on the secretion of sHLA has been studied. The molecular mass of the secreted sHLA (detected using [^{14}C]leucine) is compared with the classical transmembrane proteins.

1 Introduction

Class I human major histocompatibility complex antigens HLA are a family of proteins formed by the non-covalent association of a 43-kDa polymorphic glycoprotein encoded by the MHC termed α chain and the 12-kDa invariant chain β_2-microglobulin (β_2m) [1, 2] which is not encoded by the MHC. Although they have been traditionally considered as integral membrane proteins present in most nucleated cells [3], their presence has also been detected in the sera of mice [4], rats [5] and humans [6–8]. In humans, a concentration of about 1 μg/ml is considered normal and it increases several times in AIDS patients [9].

The source of these soluble molecules is uncertain and while some authors accept shedding from cell membranes as high molecular weight aggregates and catabolism of cell membranes by proteases [7] as the most plausible explanations, others [8, 10, 11] have shown the occurrence of different patterns of RNA splicing giving rise to class I antigens that do not interact with the membrane. Here we describe the finding that the predominant forms of HLA in serum are molecules recognized by the mAb W6/32 which detects a common determinant in all HLA-(A, B, C) [12] and polyclonal or monoclonal anti-β_2m [13], but are not recognized by mAb against A or B specificities. Such molecules can also be detected in the SN of lymphocyte cultures and their secretion increases upon stimulation by mitogens. Their presence in spleen membranes and their release in the absence or presence of protease inhibitors or chelating agents have also been studied.

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Abbreviations: **β_2m:** β_2-Microglobulin **PO:** Peroxidase

2 Materials and methods

2.1 Antibodies

The following antibodies were used in this work: mAb W6/32 [12] that recognizes a common determinant in all HLA-(A, B, C) molecules, was purchased from Serotec (Bicester, Oxon, GB). mAb anti-β_2m [13] was a gift from Dr Sánchez-Madrid, Hospital de la Princesa, Madrid, and was conjugated to horseradish peroxidase (PO) in our laboratory by the method of Nakane et al. [14]. mAb MA2.2 (specific for HLA-A2) and mAb anti-HLA-B7, B40 (cat N.092), were purchased from Atlantic Antibodies, Scarborough, ME. Polyclonal antibody against human β_2m was raised in rabbits in our laboratory. Goat anti-rabbit IgG labeled with PO was from Nordic (Tilburg, The Netherlands).

2.2 Affinity chromatography and autoradiography

Sepharose mAb W6/32 was prepared by incubating CNBr-activated Sepharose with purified mAb W6/32 according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). The final material contained 5 mg of mAb W6/32/ml of gel. Sepharose-bound human IgG was prepared by coupling human IgG with CNBr-Sepharose as above. The final material contained 15 mg of human IgG/ml of gel. Autoradiography was carried by exposing the dried gel for 30 days on Kodak X-O-MAT X-ray film between Kodak X-AR intensifying screens (Kodak, Rochester, NY).

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2.3 Miscellaneous

Sephacryl S-200 and Sepharose-2B were from Pharmacia; microELISA plates were from Dynatech (Alexandria, VA), o-phenylene diamine (OPD) and other chemicals were from Sigma (St. Louis, MO), Calbiochem (La Jolla, CA) or Merck (Darmstadt, FRG). SDS, Tween 20, Triton X-100, sodium cholate were purchased from Sigma.

2.4 Assay for HLA

Total HLA was measured by a sandwich ELISA involving the recognition of the α chain by the mAb W6/32 bound to the microtiter well, followed by the recognition of β_2m by anti-β_2m mAb labeled with PO. After the addition of substrate (OPD) the amount of HLA was proportional to the color developed (492 nm). The standardization of the method has already been published [15, 16]. Papain-solubilized HLA, purified as already described [15], was used as standard. Specific HLA was measured by substituting mAb W6/32 by mAb MA2.2 (specific for HLA-A2) or mAb 092 (specific for HLA-B7, B40) and substituting the mAb PO-anti-β_2m by a rabbit polyclonal anti-β_2m antibody and a goat anti-rabbit IgG labeled with PO as second antibody. The last modification was introduced to obtain sensitive and reproducible results. The lack of recognition of the HLA with the anti-β_2m mAb when the α chain was recognized by the specific (anti-HLA-A2 or -B7) mAb, may indicate that the determinants recognized by these mAb are in the vicinity to the determinants recognized by the anti-β_2m mAb used and thus are sterically inhibited.

2.5 Cell purification

Human PBL were prepared from healthy donors by Ficoll-Hypaque (Pharmacia) density centrifugation of heparinized blood [17]. T and non-T (B) lymphocyte fractions were separated by density sedimentation of spontaneous rosettes formed by T lymphocytes and SRBC treated with neuraminidase as previously described [18].

2.6 Culture conditions

Triplicate cultures containing 10^6 cells/ml were set up in 96-well flat-bottom plates in a final volume of 250 µl/well of RPMI 1640 supplemented with 2 mM, L-glutamine, antibiotics (gentamicin 40 µg/ml, cloxacilin 125 µg/ml, ampicilin 125 µg/ml) and 10% FCS and incubated at 37 °C with 5% CO_2. After 4 days, cell pellets and SN were collected separately by centrifugation at 1500 × g for 10 min. When used, mitogens were added at the concentrations indicated in the legends of the figures.

2.7 Metabolic labeling of cells

Cells (2.5×10^5 in 0.25 ml of the above described medium) were cultured for 36 h in the presence of 30 µg/ml of *Staphylococcus aureus* Cowan strain I (SAC, Calbiochem, La Jolla, CA) and then transferred to a leucine-free medium supplemented with 50 µCi (= 1.85 MBq) of [^{14}C]leucine (sp. act. = 300 mCi/mmol, Amersham Int., Bucks., GB). After 18 h of culture, cell pellets and SN were collected separately by centrifugation at 1500 × g for 10 min.

3 Results and discussion

3.1 Sephacryl S-200 gel filtration of human serum

The Sephacryl S-200 gel filtration of a serum from a healthy volunteer (HLA-A2, HLA-B7) is shown in Fig. 1. When the fractions are assayed for HLA by means of a sandwich ELISA [15, 16] involving the recognition of the α chain by the mAb W6/32 and a polyclonal rabbit anti-β_2m antibody, two peaks of HLA corresponding to a molecular mass of 50–60 kDa and around 200 kDa can be detected. In different individuals the amount of HLA (50–60 kDa) is variable but always > 50% and can reach as much as 80% of the total HLA present in serum. If the same assay is run substituting the mAb W6/32 by mAb specific for HLA-A2 or HLA-B7, only a peak of around

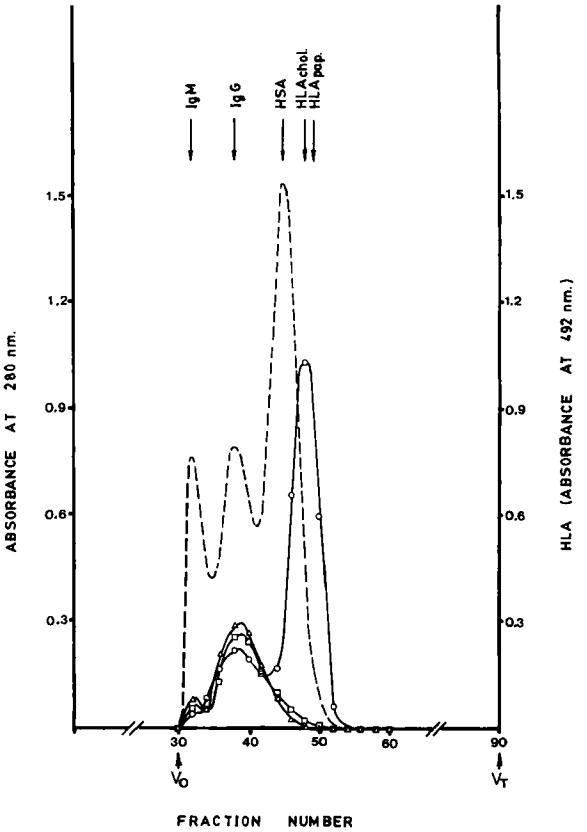


Figure 1. Sephacryl S-200 of human serum. Column: Sephacryl S-200 (2.5 × 57 cm) equilibrated in K_2HPO_4/KH_2PO_4 0.01 M 7.0, 0.15 M NaCl (PBS); flow rate: 50 ml/h; fractions: 3 ml; Sample: 2 ml of human serum (HLA-A2, HLA-B7) diluted 1:1 in PBS. Proteins are expressed as absorbance at 280 nm (-----). HLA was measured by a sandwich ELISA involving the recognition of the α chain by mAb W6/32 (○—○) or mAb MA2.2 specific for HLA-A2 (□—□) or anti-HLA-B7 mAb, B40 (Δ–Δ), and the recognition of β_2m by a rabbit polyclonal antibody. The standardization of the method has been already published [15, 16]. The arrows represent molecular mass markers. IgM (900 kDa), IgG (160 kDa), HSA (67 kDa) and HLA solubilized by sodium cholate from spleen membranes (HLA chol, 55 kDa) and HLA solubilized by digestion with papain (HLA pap, 45 kDa).

200 kDa is detected. Occasionally a third peak appearing in the void volume containing A and B specificities is also seen but it never reaches more than 5% of the total HLA. These results indicate the presence in serum of HLA molecules that do not bear A or B specificities. It is not very likely that these non-A or -B HLA molecules are proteolytic fragmentation products of the traditional HLA molecules, taking into account that their M_r is higher than papain-solubilized HLA that still maintains its allospecificity.

The nature of the 200-kDa species bearing HLA-A2 or HLA-B7 allospecificities has not been investigated in detail because such molecules have already been detected by other authors who have identified, in the high-density lipoprotein fraction of serum, molecules of HLA associated with lipids with a molecular mass of 190 kDa as estimated by gel filtration [19–21]. The presence of these molecules in the serum has been generally explained by shedding from cell membranes in association with lipids and/or other proteins.

3.2 Release of soluble class I molecules from human spleen membranes

To investigate if such molecules were also present in lymphocytes, spleen membranes from autopsy (HLA-A2, HLA-B7) were used in the next experiment. Crude membranes obtained by differential centrifugation between 12000 × g and 100000 × g, were resuspended and filtered through a column of Sepharose 2B and the fractions collected assayed for HLA as before. Fig. 2A shows the spontaneous release of HLA non-A and -B during 2 h at 4 °C. While all HLA-A2 or -B7 is detected in the void volume, indicating the presence of particulate material, about 20% of the total HLA is found as soluble molecules. When the membranes are incubated at 37 °C, the amount of soluble HLA released increases proportionally with the incubation time for a period of about 30 min, after which, a plateau is reached (data not shown). Roughly 50% of the total HLA is released upon incubation at 37 °C but < 5% of the soluble HLA bears A or B specificities. Fig. 2B shows the results obtained when membranes incubated 30 min at 37 °C were filtered through a Sepharose 2B column. As shown in Fig. 2C, these results are not influenced by the addition of protease inhibitors during the incubation, indicating that, unless a specific protease (not inhibited by the inhibitors used in the assay) is present, other mechanisms should be postulated to account for the existence of sHLA. The effect of the temperature on the release of sHLA suggests the involvement of an enzyme in the process; however, the temperature may also induce changes in the fluidity of the membrane resulting in the release of loosely bound proteins.

3.3 Effect of chelating agents on the release of sHLA from isolated membranes

The effect of different chelating agents on the spontaneous release of sHLA from membranes incubated at 37 °C is shown in Fig. 3. The order of effectivity is o-phenanthroline > EDTA > citrate > EGTA. These results provide a further support for a release mechanism involving the action of an enzyme which would need a metal ion to be active. While EDTA, EGTA and citrate behave as reversible inhibitors and can be washed away by centrifugation of the membranes, the effect of o-phenanthroline can only be partially reversed when Zn^{2+} (1 mM) is added to the washed membranes (30% recovery of the original amount released by the membranes). Other ions (Ca^{2+}, Mg^{2+}) had no effect in restoring the ability of the membranes to release sHLA. Higher concentrations of Zn^{2+} could not be assayed because this ion can induce the dissociation of sHLA into its subunits, as we have already published for the classical class I HLA [16].

3.4 Secretion of soluble class I molecules by lymphocytes in culture

The presence of secreted HLA molecules in the SN of lymphocyte cultures has already been detected by other authors [8, 11]; however, the lack of quantitative information makes it very difficult to ascertain if these molecules are really secreted, membrane shed or released into the medium upon cell death. As shown in Table 1, lymphocytes in culture secrete very small amounts of HLA (6 ± 3 ng sHLA/10^6 PBL) while they contain

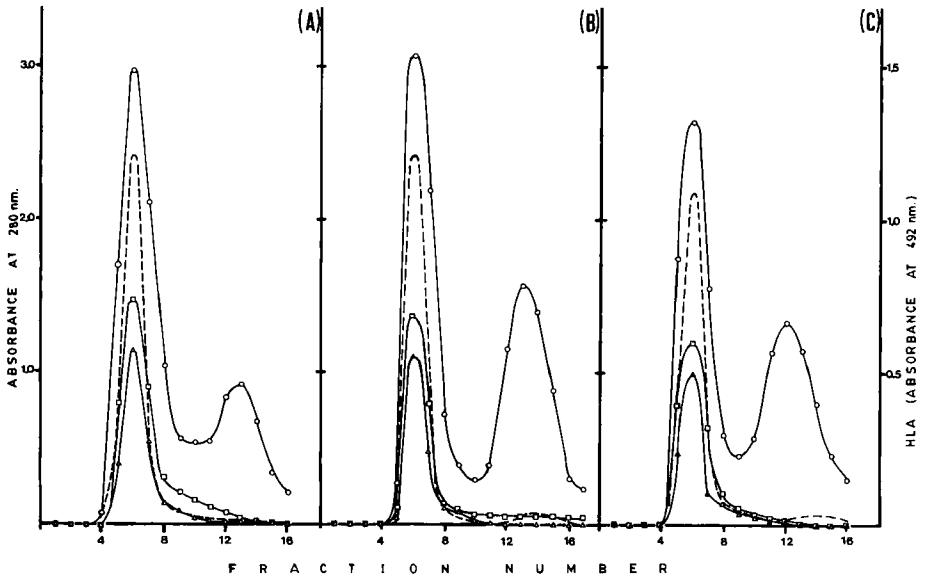


Figure 2. Sepharose 2B gel filtration of human spleen membranes. Column: Sepharose 2B (1.2 × 14 cm) equilibrated at 4 °C in Tris-HCl, 0.01 M, pH 8.0; flow rate 15 ml/h; fractions: 0.7 ml. Samples: (A) 0.5 ml of spleen membranes (5 mg/ml) obtained by differential centrifugation between 12000 × g and 100000 × g and resuspended in 0.01 M Tris-HCl after standing at 4 °C for 2 h; (B) same as above but incubated at 37 °C for 30 min; (C): same as above but incubated in the presence of 0.1 mM PMSF and 10 mM N-aminohexanoic acid. Proteins are expressed as absorbance at 280 nm, HLA was measured as described in Sect. 2.4 and represented as absorbance at 492 nm. HLA detected by the mAb W6/32 (○—○), mAb anti-HLA-A2 (□—□) or mAb anti-HLA-B7 (△—△).

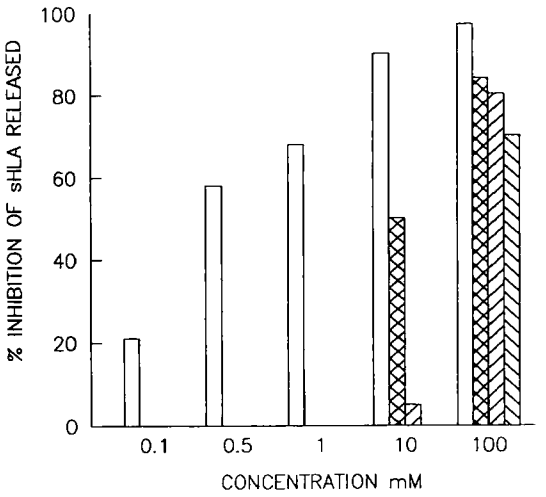


Figure 3. Effect of chelating agents on the release of sHLA from isolated membranes. Membranes (5 mg/ml) in 0.01 M Tris-HCl, pH 7.5, were incubated at 37 °C for 30 min in the presence of increasing concentrations of the following agents: o-phenanthroline (□), EDTA (⊠), EGTA (▨) and sodium citrate (▨). All these agents were previously neutralized to pH 7.5. After incubation, the membranes were centrifuged at 100000 × g for 30 min and the amount of sHLA was determined in the SN as described in Sect. 2.4. The results are expressed as % inhibition of the released sHLA. The total sHLA released by the membranes (0% inhibition) in the absence of chelating agents was 32 μg/ml.

an appreciable amount of HLA bond to the membrane (21.4 ± 1.9 ng). However, if activation is induced by the presence of PHA the secretion of sHLA increases by a factor of 20 (104 ± 16 ng) while the amount of HLA bound to the membrane increases only by a factor of two to three (51.1 ± 4.9 ng). These results argue against membrane shedding or release after death as the mechanisms responsible for the sHLA accumulation in the SN of lymphocytes in culture. This secretion can be induced by both T and B cell mitogens, indicating that this could be a general phenomenon in lymphocyte activation.

Table 1. Effect of different agents on the secretion of HLA by lymphocytes in culture[a)]

	Total HLA (ng/ml)	
	Soluble	Cell bound
PBL/ml	6 ± 3	21.4 ± 1.9
PBL + PHA	104 ± 16	51.1 ± 4.9
T lymphocytes	4 ± 2	ND
T lymphocytes + PHA	123 ± 24	ND
T lymphocytes + PWM	77 ± 20	ND
T lymphocytes + PMA + IO	23 ± 4	ND
B lymphocytes	3 ± 2	ND
B lymphocytes + SAC	44 ± 5	ND
B lymphocytes + PMA	38 ± 5	ND

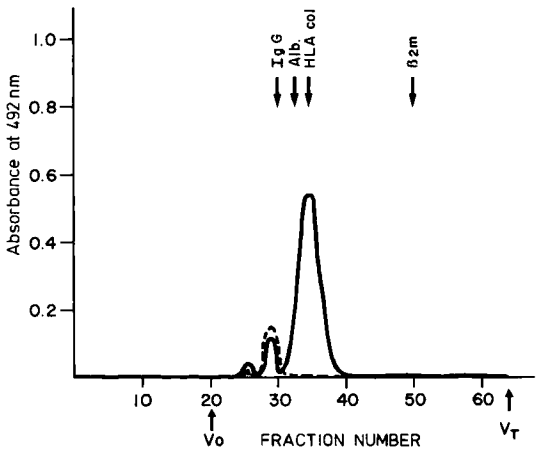
a) PBL or isolated B or T lymphocytes were cultured at a concentration of 10^6 cells/ml in 150-μl aliquots in 96-well culture plates as described in Sect. 2.6. After incubation for 96 h the plates were centrifuged (1500 × g, 10 min) and aliquots (5–50 μl) were used to quantify HLA in the SN as already described. Cell-bound HLA was estimated after solubilization of the cells in 1% Triton X-100. Values represent the mean ± 2 SD of seven experiments. Papain-solubilized HLA [15] was used as standard. When used, the final concentration of the following compounds were: PHA 36 μg/ml; PWM final dilution 1 : 1000; SAC 30 μg/ml; PMA 10^{-7} M; ionophore A23187 (IO) 5 μg/ml.

3.5 M_r determination of class I molecules secreted by lymphocytes in culture

As in the above experiments, the secreted sHLA behaves as a 50–60-kDa molecule by gel filtration on Superose 12 (Fig. 4) and can be recognized by the mAb W6/32, but only traces of HLA-A2 or -B7 can be detected by their corresponding mAb and always at a higher molecular mass (about 200 kDa) in the SN of cells (HLA-A2, HLA-B7) in culture. The nature of the 200-kDa species bearing HLA-A2 or HLA-B7 specificities has not been investigated in detail because of the very small amounts present in the SN of cells in culture (< 5% of the total sHLA). We believe they represent shedding products of the classical HLA complexed with lipids and/or other proteins.

3.6 SDS-PAGE analysis of sHLA

The M_r determinations by SDS-PAGE of the sHLA released from membranes purified by affinity chromatography on mAb W6/32-Sepharose are shown in Fig. 5 A (lane 1). For comparison, the remaining HLA bound to the membrane solubilized by sodium cholate and purified in the same way is shown in lane 3. As can be observed both molecules have a very similar M_r. If 43 kDa is the molecular mass calculated for the α chain of the detergent-solubilized HLA, that for the heavy chain of the sHLA (non-A or -B) can be estimated to be 41–42 kDa. Fig. 5 B shows the SDS-PAGE analysis of the secreted HLA (lane 1) and the membrane-bound molecules (lane 2) when B lymphocytes stimulated with SAC were cultured in the presence of [^{14}C]leucine, demonstrating again that both types of molecules have very similar M_r.



Figue 4. M_r determination of sHLA by lymphocytes in culture. Column: Superose 12 (FPLC, Pharmacia) 1.5 × 30 cm equilibrated in PBS; sample: the SN (1 ml) of 10^6 cells (homozygous for HLA-A2) cultured in the presence of PHA (36 μg/ml) for 4 days was concentrated to 0.1 ml by lyophilization and injected into the above column. Fractions of 0.35 ml were collected (flow rate: 20 ml/h). HLA was measured as described in Sect. 2.4 and represented as absorbance at 492 nm. HLA detected by the mAb W6/32 (——) or anti-HLA-A2 mAb (-----).

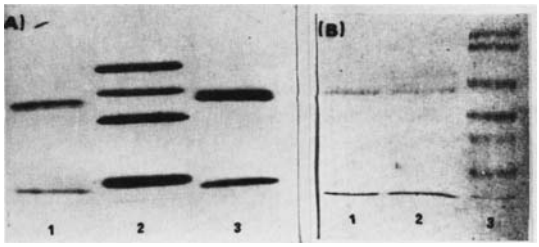


Figure 5. SDS-PAGE analysis of soluble and membrane-bound HLA. (A) Polyacrylamide gel (12%) run as described by Laemmli (26). Lane 1: HLA spontaneously released (sHLA) from spleen membranes upon incubation for 30 min at 37 °C in the presence of protease inhibitors (see Fig. 2 C) and purified by affinity chromatography on W6/32-Sepharose. Lane 2: protein standards: BSA (66 kDa), OVA (45 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12 kDa), Lane 3: HLA remaining bound to membranes after incubation at 37 °C. Procedure: 5 ml of crude membranes (10 mg/ml) prepared as described in Fig. 2 were incubated for 30 min at 37 °C. After centrifugation for 1 h at 100 000 × g the SN was filtered through a column (1 ml) of human IgG-Sepharose (15 mg/ml of gel). The filtrate was then absorbed onto a 0.5-ml column of W6/32-Sepharose (5 mg/ml of gel) and extensively washed with 0.5 M NaCl, 0.2% Triton X-100, 0.01 M Tris-HCl, pH 8.0 (20 ml) followed by a final wash with 0.01 M Tris-HCl, pH 8.0 (5 ml). HLA bound to the column was then eluted with 5 volumes of 0.1 M diethylamine, pH 11.5, lyophilized and resuspended in sample buffer (2% SDS, 0.01 M 2-ME, 0.05 M Tris-HCl, pH 6.8, 0.01% bromophenol blue and 10% glycerol). The 100 000 × g pellet was resuspended in 5 ml of Tris-HCl, pH 8.0, containing 2% sodium cholate and centrifuged again at 100 000 × g. The solubilized HLA was then purified in the same way as described above for the sHLA. (B) SDS-PAGE of metabolically labeled HLA. Lane 1: autoradiography of HLA secreted by B lymphocytes stimulated by SAC. Lane 2: autoradiography of HLA remaining bound to the membrane of B lymphocytes stimulated by SAC. Lane 3: pre-stained standards (Bio-Rad, Richmond, VA; 130 kDa, 75 kDa, 50 kDa, 39 kDa, 27 kDa, 17 kDa). Procedure: Cells (2.5×10^5) were cultured in the presence of [^{14}C]leucine as described in Sect. 2.7. The secreted HLA contained in the SN was purified by affinity chromatography as described above. The cells were then lysed in 1 ml of hypotonic medium (0.01 M Tris-HCl, pH 8.0) and centrifuged at 100 000 × g for 1 h. The pellet was then solubilized with 1% Triton X-100 and the HLA bound to the membranes was purified as before.

4 Concluding remarks

Class I molecules behaving similarly to the ones described here, have been reported in mice ([22, 23]; Qa antigens). In humans, the sequence of non-HLA-A, -B, -C class I MHC genes in mutant lymphoplastoid cell lines has been recently reported [19, 24]. An important question that still remains to be answered is the way by which these molecules interact with the membrane. HLA (A, B, C) are known to be transmembrane proteins and some Qa-like proteins seem to be anchored through a phosphatidylionositol tail [25] and can be released by the action of phospholipases C or D. In our case the fact that these sHLA molecules can be spontaneously released upon incubation at 37 °C does not speak for the action of phospholipases C or D, although the possibility that during the incubation an endogenous phospholipase present in the membrane is acting on these molecules can not be excluded. Experiments dealing with the amino acid sequence of these proteins and the analysis of lipids would undoubtedly help to explain the nature of these molecules.

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