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Soluble HLA class I antigen secretion by normal lymphocytes: relationship with cell activation and effect of interferon-gamma

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SUMMARY

HLA class I antigens are thought to be integral membrane proteins. However, soluble forms of these molecules have been detected. Our laboratory has recently shown that the predominant form of these soluble proteins present in human serum, spleen tissue and culture supernatant of activated lymphocytes exhibits molecular weight and structure similar to classical HLA class I antigens, but lacks HLA A or B polymorphic determinants. In the present study, the secretion of such soluble proteins by lymphocytes has been further explored. Phytohaemagglutinin-stimulated normal lymphocytes secrete considerable quantities of soluble HLA (sHLA) class I proteins. This secretion seems to be a general property of lymphocytes, since activation of T as well as B cells by appropriate mitogens equally induce sHLA I secretion. Lymphocytes require RNA and protein synthesis, but not DNA synthesis, for the secretion to occur. Kinetic studies reveal that maximal sHLA I secretion precedes the peak of DNA synthesis by 24 h. *In vitro* stimulation with antigens or alloantigens also provokes sHLA I secretion. Moreover, this phenomenon has also been detected for *in vivo*-activated lymphocytes, as enhanced spontaneous sHLA I secretion was observed in cultures of low-density blastic B and T cells, and of blood lymphocytes obtained from normal subjects who had received a booster immunization 5 days earlier. Interferon-gamma (IFN-γ) increases the expression of membrane-bound class I antigens but does not induce any sHLA I secretion, suggesting that both molecules are under different regulatory mechanisms. Our results indicate that human lymphocytes, upon stimulation, actively secrete considerable amounts of a soluble form of these biologically relevant proteins.

Keywords soluble HLA class I antigens HLA class I antigen secretion human lymphocyte activation regulation of HLA class I interferon-gamma

INTRODUCTION

MHC class I antigens are heterodimeric glycoproteins consisting of a 43-kD alpha chain, and a 12 kD chain, termed beta-2-microglobulin (β2-m), which are not covalently bound (Kimball & Coligan, 1983). These molecules are present on the surface of many different cell types (Ploegh, Orr & Strominger, 1981) and as integral proteins of the cell membrane they appear to be involved in important mechanisms of immune intercellular contact (Zinkernagel & Doherty, 1979). Soluble forms of class I antigens have been demonstrated in several species including humans (Kress *et al.*, 1983; Pellegrino, Ruso & Allison, 1984; Lew, Maloy & Coligan, 1986; Krangel, 1986, 1987; Robinson, 1987; Spencer & Fabre, 1987; Singh, Brown & Roser, 1988; Dobbe *et al.*, 1988). We have recently described that the soluble form of HLA class I molecules (sHLA I) predominant in human serum, spleen tissue and supernatant of lymphocyte cultures has a molecular weight of about 55 kD, and consists of a 40–42 kD alpha chain bound to β2-m. Such sHLA I can be recognized by monoclonal antibodies (MoAbs) reactive with class I monomorphic determinants (W6/32) and with β2-m, but not with MoAbs reactive with polymorphic A and B determinants (Villar *et al.*, 1989). The nature of these soluble non-A, non-B HLA class I proteins is not understood and some of the non-classical HLA class I genes recently described (Srivastava *et al.*, 1987; Geraghty, Koller & Orr, 1987; Paul *et al.*, 1987; Shimizu *et al.*, 1988) might code for such molecules.

In the present study, the secretion of sHLA I by normal lymphocytes has been further investigated. T and B lymphocytes secrete considerable amounts of sHLA I to the culture supernatants in a variety of *in vitro* and *in vivo* activation systems. In addition, interferon-gamma (IFN-γ) enhanced the expression of membrane-bound HLA class I antigens while not affecting sHLA I secretion, suggesting a different regulatory

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mechanism for both kinds of class I molecules. This novel secretory system might be of importance in human lymphocyte biology.

MATERIALS AND METHODS

Materials

Percoll was purchased from Pharmacia (Uppsala, Sweden). *Staphylococcus aureus* Cowan I (SAC), hydroxiurea (OHU), actinomycin D (AcD) and ionophore A23187 (IO) were from Calbiochem (San Diego, CA). TPA was from Sigma Chemical Co. (St Louis, MO). Phytohaemagglutinin (PHA) was obtained from Wellcome Diagnostics (Bedford, UK). OKT3 MoAb was obtained form Ortho Diagnostic Systems (Raritan, NJ). Anti-T cell receptor $\alpha\beta$ TcR MoAb WT/31 was from Sambio (Uden, The Netherlands). Lipopolysaccharide (LPS) was obtained from Difco Laboratories (Detroit, MI). MoAb Leu11b (CD16) was from Beckton Dickinson (Mountain View, CA). Human recombinant IFN-γ was supplied by Boehringer (Mannheim, FRG). *Candida albicans* dermatophitin 'O' was obtained from Hollister (Berkeley, CA). Tetanus toxoid was a generous gift of Laboratorios Llorente (Madrid, Spain). M24-AR Dynatech microtitre plates were used in the ELISA. The 96-well flat-bottomed microtitre plates used in cell cultures were provided by Costar (Cambridge, MA). W6/32 MoAb, which recognizes a monomorphic determinant of HLA class I antigens (Bernstable *et al.*, 1978) and FG2/2 MoAb, which recognizes human β2-m (Bernabeu *et al.*, 1986) were generously supplied by Dr F. Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain). Goat anti-mouse IgG labelled with peroxidase was from Nordic (Tilburg, The Netherlands). ^{3}H-thymidine (^{3}H-TdR) with a specific activity of 5 Ci/mM was purchased from Amersham International (Amersham, UK). Goat F(ab')$_2$ anti-human IgM antibodies and FITC-conjugated goat F(ab')$_2$ anti-mouse IgG antibodies were provided by Tago (Burlingame, CA). Crude B cell growth factor (BCGF) was obtained from Sera-Lab (UK).

Cell preparation and separation

Human peripheral blood mononuclear cells (PBMC) were prepared by Ficoll–Hypaque density centrifugation of heparinized blood obtained from healthy volunteers (Boyum, 1968). In some experiments, blood was obtained from volunteers before and 5 days after a conventional tetanus toxoid (5 Lf, intramuscular administration) booster immunization. Tonsilar tissue was obtained from subjects undergoing tonsillectomy for chronic tonsillitis. Tonsil and blood T and non-T (B) lymphocyte fractions were separated by density sedimentation of spontaneous rosettes formed by T lymphocytes and sheep erythrocytes pretreated with neuraminidase (De la Sen, Garcia-Alonso & Brieva, 1986). In some experiments, tonsil and blood mononuclear cells were depleted of natural killer (NK) cells by treatment with Leu11b MoAb and complement (Brieva, Targan & Stevens, 1984). NK cell-depleted populations were separated into B and T cells as indicated above, and fractionated on a discontinuous Percoll gradient (De la Sen *et al.*, 1986). Blood neutrophils were obtained by density centrifugation on Mono-Poly Resolving Medium (Flow Laboratories, Irvine, UK). This cell preparation contained 90–95% neutrophils. Monocytes were obtained from blood by incubating PBMC at 2×10^6/ml on plastic Petri dishes at 37°C for 2 h. Adherent populations were recovered by using a rubber policeman, and consisted of 75–90% monocytes as determined by positive non-specific esterase staining. Cultures of purified T cells and T cell fractions were supplemented with 10% autologous monocytes.

Cell culture

All cell preparations were adjusted at 1×10^6/ml, and triplicate cultures were set up in a final volume of 0·25 ml/well in 96-well flat-bottomed plates. The cultures were incubated at 37°C with 5% CO_2 for 4 days, unless otherwise stated. The culture medium consisted of RPMI 1640 supplemented with L-glutamine (10 mM), gentamycin (0·05 mg/ml), and 10% fetal calf serum (FCS). Activators and antigens were added to the cultures at the indicated concentration (that had been previously found to be optimal). Cell-free supernatants were obtained at the end of the culture period by centrifugation at 1500 ***g*** for 10 min and stored at −20°C. To evaluate DNA synthesis, 1 μCi of ^{3}H-TdR was added to certain cultures for the last 16 h of incubation, and then the cells were harvested on glass/fibre filters and counted by liquid scintillation spectrometry.

Analysis of the kinetics of HLA class I antigen cellular distribution

PBMC (10^6 cells/ml) were cultured in the absence (control) and in the presence of PHA or IFN-γ. Aliquots of these cultures were obtained at 24, 48, 72, and 96 h of culture period, and were immediately processed. Three different fractions of every aliquot were then prepared as follows: cell-free supernatant were collected as described above (referred as to secreted HLA); subsequently, the cell pellets were resuspended in hypotonic medium (20 mM Tris pH 8·0), freeze-thawed three times, centrifuged at 100 000 ***g*** for 1 h, and the supernatants collected (referred as to cytoplasmic HLA); and the pellets were solubilized with 1% final concentration of sodium cholate in 20 mM Tris, pH 8·0, for 15 min at 37°C, and centrifuged again as before. These final supernatants were then collected (referred as to membrane HLA). All the fractions were kept at −20°C until testing for their content in HLA class I antigens by ELISA.

ELISA for the quantification of HLA class I proteins

Total HLA class I antigens were measured by a sandwich ELISA involving the recognition of the alpha chain by the MoAb W6/32 bound to the micro-ELISA well, followed by the recognition of β2-m by MoAb FG2/2 labelled with peroxidase. After the addition of the substrate *o*-phenylenediamina (OPD), the amount of HLA class I was proportional to the colour developed at 492 nm. The standardization of the method has been published (Ferreira *et al.*, 1983; Revilla *et al.*, 1986). HLA class I antigens solubilized by papain and purified, as already described (Ferreira *et al.*, 1983), were used as standard.

FACS analysis of membrane HLA class I antigens

PBMC were cultured in the presence and in the absence of PHA or IFN-γ. After 3–4 days of incubation, cells were harvested, and the HLA class I antigens present on the cell membrane was evidenced by an indirect immunofluorescence technique. In brief, cells (1×10^6 cells/100 μl) were incubated with 10 μg of W6/32 MoAb for 30 min at room temperature, washed, and membrane-bound MoAb was revealed with FITC-conjugated goat F(ab')$_2$ anti-mouse IgG antibodies. Labelled populations were monitored in an EPICS Profile (Coulter Electronics, Hialeah, FL).

RESULTS

In vitro *activation of human lymphocytes induces sHLA class I antigen secretion*

In a first series of experiments, normal PBMC were cultured in the presence or absence of PHA, and the amount of sHLA I antigens present in the supernatant after 4 days of incubations was quantified by ELISA. As shown in Fig. 1a, very little sHLA I was detected in the supernatant of non-stimulated cultures. PHA induced a 20 times mean increase of the sHLA I secretion. Such secretion strictly depended on *de novo* RNA and protein synthesis, since the addition of actinomycin D and cycloheximide to the cultures provoked more than 90% inhibition of the secretion. However, DNA synthesis was not required for this phenomenon to occur, as the addition of hydroxiurea at concentrations which reduced over 90% of the proliferative response to PHA did not affect sHLA I secretion in the same cultures.

Since PBMC preparations consist of a complex cellular mixture, experiments were performed in order to determine the source of the sHLA I secreted in PBMC cultures. Enriched populations of monocytes or neutrophils were cultured for 4 days in the absence or presence of PHA, TPA plus ionophore or LPS. Only background quantities of sHLA I were detected in the supernatants of such cultures. Conversely, as shown in Fig. 1b, T lymphocyte preparations could be stimulated to secrete large amounts of sHLA I by PHA, anti-CD3 and anti-Ti MoAbs, and, to a lesser extent, by TPA plus ionophore (seven times or more above the control). Purified populations of $CD4^+$ and $CD8^+$ T lymphocytes also secreted sHLA I when stimulated with PHA (data not shown). Similar results were observed when enriched preparations of B lymphocytes were incubated in the presence of SAC, anti-IgM plus BCGF, or TPA (Fig. 1c).

The kinetics of sHLA I secretion expressed as daily increments and of DNA synthesis estimated by ^{3}H-TdR incorporation by non-stimulated and PHA-activated PBMC cultures were next investigated. Figure 1d shows that PHA-induced sHLA I secretion in PBMC cultures was demonstrable as soon as 24 h (five-fold above background), and reached a maximum at 72 h, preceding the peak of DNA synthesis by 24 h. After 3 days of culture, the sHLA I secretion clearly declined. Similar kinetics were observed when cultures of SAC-induced B cells and anti CD 3-induced T cells were examined (data not shown).

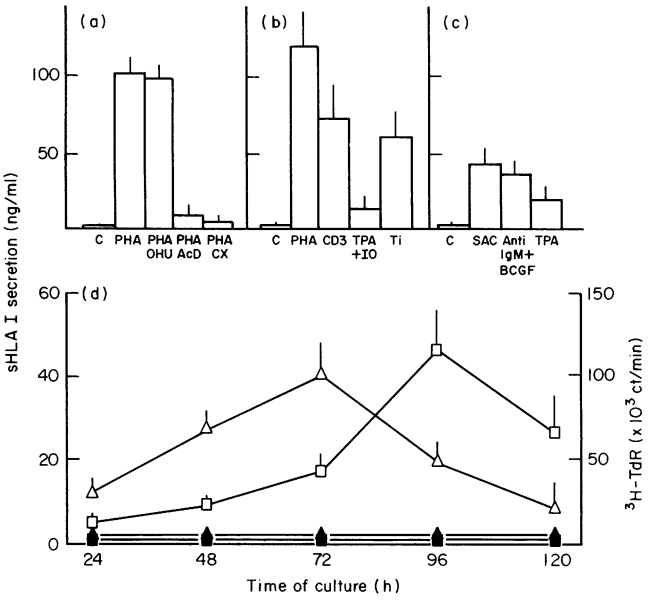

Fig. 1. Soluble HLA (sHLA I) class I antigens secretion by normal lymphocytes. Different lymphocyte preparations were cultured in the presence and in the absence of a variety of activators for 4 days, and the quantity of sHLA I molecules secreted to the culture supernatant was determined by ELISA. The cell preparations used were peripheral blood mononuclear cells (PBMC) (a and d), purified T lymphocytes (b) and purified B lymphocytes (c). The substances indicated in the figure were used at the following final concentrations: phytohaemagglutinin (PHA) at 5 μg/ml; hydroxiurea (OHU) at 0·5 mM; actinomycin D (AcD) at 5 μg/ml; cycloheximide (CX) at 10 μg/ml; TPA at 40 ng/ml; ionophore (IO) at 500 ng/ml; CD3 (OKT3 MoAb) at 1/5000 (v/v); Ti (WT31 MoAb) at 1/1000 (v/v); SAC at 1/1000 (v/v); anti-IgM antibodies at 300 ng/ml; BCGF at 1/20 (v/v). Results are expressed as the mean ± s.e.m. of different experiments: a, $n = 47$; b, $n = 9$; and c, $n = 7$. (d) Kinetic studies of sHLA I secretion (triangles) and DNA synthesis (squares) in PBMC cultured in the presence (open symbols) or absence (closed symbols) of PHA. Values were expressed as daily increment for both activities. Results represent the mean ± s.e.m. of seven different experiments.

In vitro *antigen activation induces sHLA I secretion by lymphocytes*

In order to investigate whether the sHLA I secretion was not only inducible in mitogen-triggered systems, the stimulation of PBMC preparations with antigens (tetanus toxoid or *Candida albicans* dermatophitin 'O') and alloantigens (mixed lymphocyte reaction) was also analysed. As can be seen in Fig. 2, both activation systems induced the secretion of sHLA I molecules by a mean of nine and six times, respectively.

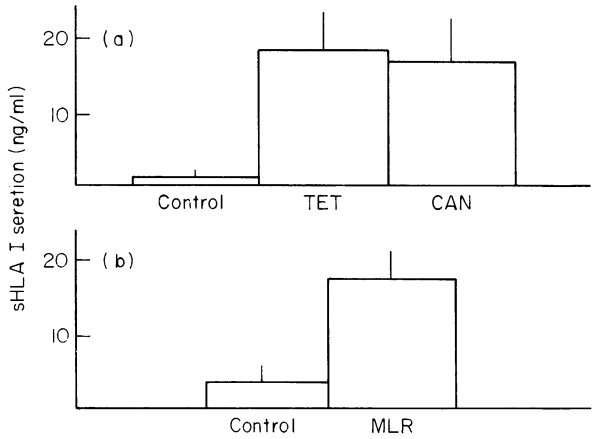

Fig. 2. Lymphocyte secretion of soluble HLA (sHLA) class I antigens in antigen- and alloantigen-driven cultures. sHLA I antigens were determined in the culture supernatants obtained after 6 days of incubation. (a) Peripherral blood mononuclear cells (PBMC) were cultured in the absence (contol) and in the presence of tetanus toxoid (TET; 5 μg/ml) or *Candida albicans* dermatophitin O (CAN; 2 μg/ml). Results are expressed as the mean ± s.e.m. of four different experiments. (b) PBMC (at 10^6 cells/ml) from pairs of hysto-incompatible donors were cultured alone or in combination. Control sHLA I secretion for each combination was estimated as the sum of sHLA I secreted by these PBMC preparations separately cultured. Such values (control) are compared with those observed in the bidirectional mixed lymphocyte reaction (MLR). Results are expressed as the mean ± s.e.m. of 11 different experiments.

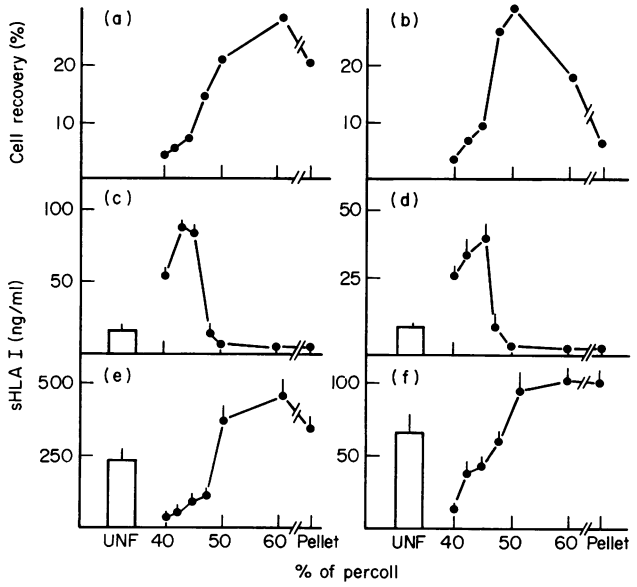

Fig. 3. Spontaneous and mitogen-induced sHLA I secretion by Percoll fractionated tonsilar B and T lymphocytes. Unfractionated (UNF) and different cell fractions were cultured in the absence and presence of phytohaemagglutinin (PHA) (T cells) and SAC (B cells), in order to explore spontaneous and mitogen-induced sHLA I secretion, respectively. Results of one out of three similar experiments are shown. (a) and (b), cell recovery; (c) and (d), spontaneous sHLA I secretion; (e) and (f), mitogen-induced sHLA I secretion. (a), (c) and (e), T cells; (b), (d) and (f), B cells. Values represent the mean ± s.e.m. of triplicate cultures.

sHLA I secretion by lymphocytes stimulated in vivo

The possibility that *in vivo* activation also elicited sHLA I secretion by lymphocytes was next examined. In this regard, it is well established that activated lymphocytes become low-density blastic cells (Defranco *et al.*, 1982; Aman, Ehling-Henrikson & Klein, 1984). Therefore, tonsil and blood B and T lymphocytes were fractionated on a discontinuous Percoll gradient, and the spontaneous (non-stimulated cultures) as well as the mitogen-induced sHLA I secretory capability of the different cell fractions were evaluated. Figure 3 shows a representative experiment. As can be seen, the minor fraction of low density T and B lymphoblasts (Fig. 3a, b, fractions of 40–45% Percoll) were capable of spontaneous secretion of sHLA I (Fig. 3c, d). No or low spontaneous activity was detected in cultures of denser T and B cells which, in contrast, contained most of the lymphocytes responsible for mitogen-induced sHLA I secretion (Fig. 3e, f).

Booster immunization leads to the transient release of activated lymphoblasts into the circulation (Thompson & Harris, 1977; Ehrnst, Lambert & Fagraeus, 1978) and, in accordance, the spontaneous sHLA I secretion by non-stimulated cultures of lymphocytes obtained prior to and 5 days after a conventional booster immunization were compared. Lymphocytes obtained after *in vivo* challenge showed an increased capability of spontaneous sHLA I secretion when compared with pre-booster samples ($20{\cdot}4 \pm 6{\cdot}5$ *versus* $3{\cdot}2 \pm 1{\cdot}3$, mean ± s.e.m., $n=5$; $P<0{\cdot}0025$). Spontaneous sHLA I secretion observed in these experiments was also actively carried out by the cells, as a 90% inhibition was obtained by adding cycloheximide to the cultures.

Effect of IFN-γ on the secretion and membrane expression of HLA class I antigens

IFN-γ is a well-known inducer of HLA class I antigen expression on the membrane of many cells including lymphocytes (Heron, Hockland & Berg, 1978). We therefore asked whether such an agent had any effect on the secretory phenomenon described above. Figure 4 shows that IFN-γ markedly enhanced HLA class I antigen expression on the lymphocytic membrane, as determined by FACS analysis of W6/32 MoAb reactivity (Fig. 4a) or by ELISA (Fig. 4c). However, this cytokine was unable of inducing sHLA I secretion by lymphocytes (Fig. 4b). Nevertheless, PHA increased both sHLA I secretion (Fig. 4b) and membrane HLA class I expression (Fig. 4a, c). Additionally, IFN-alpha and IFN-beta used in a wide range of concentrations (10–10000 U/ml) were also non-effective in increasing sHLA I secretion.

The failure of IFNs in enhancing sHLA I secretion by cultured lymphocytes could be interpreted as either a lack of induction or an impaired secretion of such molecules. In order to analyse further the different effect of IFN-γ on membrane and soluble class I molecules, the kinetics of total HLA class I antigen distribution in the membrane, cytoplasm and extracellular medium of non-stimulated, PHA-activated and IFN-γ-treated lymphocyte cultures were determined (Fig. 5). The upper row represents the HLA incorporated into membranes of non-stimulated (control) PBMC, or PBMC incubated in the presence of PHA or IFN-γ. These two agents increased HLA class I expression on lymphocyte membrane (Fig. 5b, c), and this is in good agreement with the results shown in Fig. 4c: the middle row shows the cytoplasmic class I content in similar cultures. A peak of cytoplasmic HLA class I proteins occurred at 48 h of culture in the presence of PHA (Fig. 5e) while lower levels could be detected in IFN-γ-treated cultures (Fig. 5f). Finally, in the bottom row, the quantities of HLA class I antigen secreted to the culture supernatant are registered. As can be observed, the secretion occurred in the presence of PHA (Fig. 5h), but not in control (Fig. 5g) or IFN-γ-treated (Fig. 5i) cultures. When comparing the results shown in Fig. 1d and Fig. 5h for the secretion of sHLA I, it should be noted that while in Fig. 1d the quantity of these molecules are represented as daily increments, in Fig. 5h the values are cumulative. This explains why in Fig. 1d a peak of secretion is found, while in Fig. 5h the same effect is seen as reaching a plateau. According to these data, IFN-γ does not seem to induce sHLA I.

DISCUSSION

We have shown that stimulated peripheral blood and tonsil cell preparations actively secrete considerable amounts of a soluble form of HLA class I antigen (sHLA I). In these cultures, lymphocytes appeared to be the main if not the only source for such molecules. Our results seem to indicate that sHLA I secretion might be a general property of lymphocytes, since B as well as T cells developed such ability following appropriate stimuli. Lymphocyte sHLA I secretion might be of physiological relevance, as this capability could be triggered by antigen-receptor-mediated signalling. Spontaneous sHLA I secretion by *in vivo* normally occurring and antigen-generated lymphocytes further supports this assumption.

The mechanism of sHLA I secretion remains to be elucidated. The fact that *in vivo*-activated lymphocytes sponta-

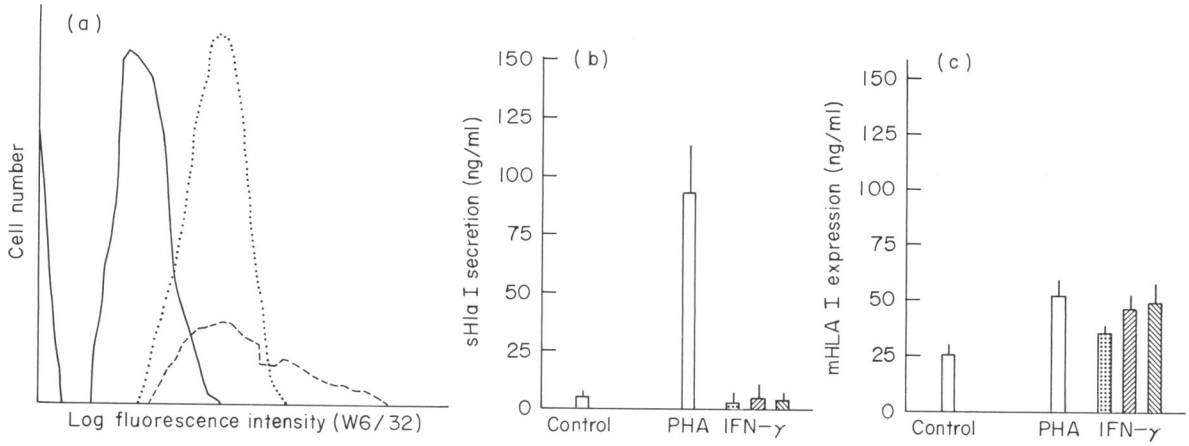

Fig. 4. Differential effect of phytohaemagglutinin (PHA) and interferon-gamma (IFN-γ) on the lymphocyte capacity of soluble HLA (sHLA) class I antigen secretion and membrane-bound HLA (mHLA) class I expression. Peripheral blood mononuclear cells (PBMC) were cultured in the absence (control) and presence of PHA or IFN-γ for 3 days. mHLA antigens were detected by FACS analysis (a) or by ELISA in solubilized cell membranes (c). sHLA I secreted to the culture was evaluated by ELISA (b). (a), results of a representative experiment. —, control, ..., IFN-γ, ---, PHA; (b) and (c), mean ± s.e.m. of four experiments; IFN concentrations: ▨, 10 U; ▩, 100 U; and ▧, 1000 U per ml.

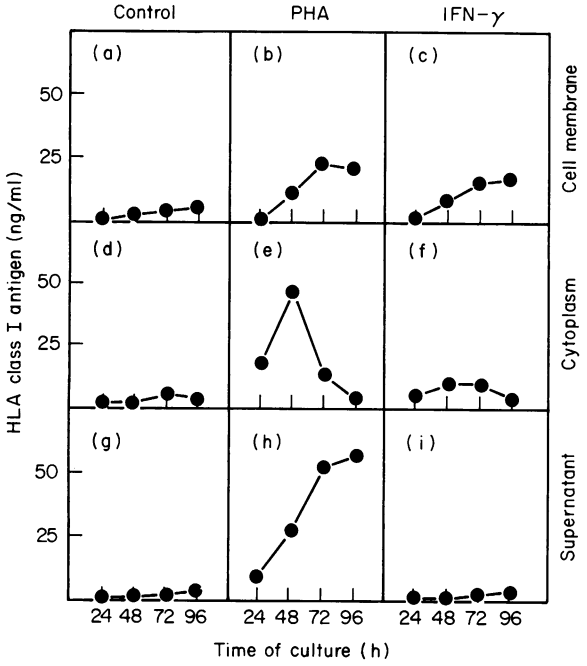

Fig. 5. Kinetics of the cellular distribution of HLA class I proteins induced by phytohaemagglutinin (PHA) and interferon-gamma (IFN-γ) in lymphocyte cultures. Peripheral blood mononuclear cells (PBMC) were cultured in the absence (control) (a,d,g) and the presence of PHA (b,e,h) or IFN-γ (500 U/ml) (c,f,i), and the content of HLA class I antigens was determined in the extracellular fluid (g,h,i), the cytoplasm fraction (d,e,f) and the cell-membrane fraction (a,b,c) at different times. Results of one out of three similar experiments are shown.

neously secreted sHLA I appears to rule out the possibility that these molecules are an artefactual by-product induced by *in vitro* manipulations. In addition, sHLA I does not seem to be the result of either passive release or membrane shedding following cellular lysis, since the generation of sHLA I was absolutely dependent on *de novo* protein synthesis; such molecules mostly showed 50–60 kD (Villar *et al.*, 1989); and maximal secretion occurred in the absence of detectable cell death as determined by trypan blue exclusion test. Non-specific proteolysis due to enzymes released to the supernatants during the cell activation process appeared neither to be involved, since substantial 'secretion' of other membrane proteins such as classical HLA class I antigens, which are known to possess an extracellular proteolitic site close to the membrane insertion region (Cresswell, Turner & Strominger, 1973) were not observed (Villar *et al.*, 1989); and sHLA I secretion increase was not seen when non-stimulated lymphocytes were co-cultured for 2 days with lymphocytes previously activated with mitogens (data not shown). These observations indicate that sHLA I are the result of an active secretory process undergone by intact and functional lymphocytes.

The events that ultimately determine the lymphocytic sHLA I secretory phenomenon are unknown. The fact that IFN-γ does not induce sHLA I secretion suggests that these soluble proteins are regulated by mechanisms different from those demonstrated for classical membrane-bound HLA class I antigens (Cresswell, 1987).

No function has been ascribed yet to soluble MHC class I antigens (Gussow & Ploegh, 1987). Lymphocyte sHLA I secretion seems to be closely connected with the cell activation process, since marked secretory activity was readily detectable in every lymphocyte activation system tested, including *in vivo* induction and *in vitro* mitogen, alloantigen and antigen stimulation. The fact that maximal sHLA I secretion preceded the peak of DNA synthesis by at least 24 h suggests that sHLA I may play some role in the lymphocyte-triggering process. This possibility is under current study in our laboratory.

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