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Control of HLA-A,B,C synthesis and expression in interferon-treated cells

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Cytofluorimetric analyses with monoclonal antibodies were used to follow the specific effect of highly purified human leucocyte interferon (IFN-α) on the expression of transplantation antigens (HLA)[1] in Molt 4, a thymus leukaemia cell line. After a lag period of ~10 h the amount of HLA steadily increases to reach a maximum at day six of ~10 times its original. The effect is reversible and due to an increase in the rate of synthesis of HLA and β2m molecules as demonstrated by analysis of [^{35}S]methionine incorporation into specific proteins after a 3-h pulse of Molt 4 cells treated with IFN-α for one or six days. A dramatic increase in the amount of mRNA that hybridised with a [^{32}P]cDNA probe containing HLA sequences has also been demonstrated. Surface iodination of IFN-treated Molt 4 cells showed an increase in the iodinated HLA and β2m chains and in addition another, apparently unrelated, component with an apparent mol. wt. of 16 000. This component, which appears after incubations with IFN-α for longer than 1 h at 37°C, was also detected in the B lymphoid cell lines RAJI and DAUDI.

Key words: HLA-A,B,C expression/interferon-α/Molt 4/mRNA levels/surface antigens

Introduction

In addition to its anti-viral activity, leucocyte interferon (IFN-α) has a number of other biological activities. These include inhibition of cellular growth (Hilfenhaus *et al.*, 1976; Stewart *et al.*, 1976; Adams *et al.*, 1975), and enhancement of expression of histocompatibility antigens (HLA) in mouse (Vignaux and Gresser, 1977; Lindahl *et al.*, 1976) and human (Fellous *et al.*, 1981; Heron *et al.*, 1978) cells. The latter effect is specific and observed with permanent cell lines. For these reasons it provides an attractive model to further our understanding of the molecular bases of the control of quantitative expression of surface antigens. While the effect of human IFN-α on human lymphoid B-cell lines and on normal peripheral blood lymphocytes (Fellous *et al.*, 1979; Heron *et al.*, 1978) has been reported, the growth inhibitory activity of IFN-α conspires against an exhaustive study of the effect on cell-surface antigen expression. Furthermore, most studies have been performed with only partially purified interferon.

Highly purified human IFN-α became available in large quantities following the derivation of the anti-interferon monoclonal antibody (McAb) NK2 (Secher and Burke, 1980). In this work we have investigated the effect of NK2-purified IFN-α on the expression of surface antigens of the T-cell line Molt 4. Molt 4 cells proved to be particularly useful for this study because, while the increase in HLA was greater than observed with other lines, even 10 000 units/ml of IFN-α had little or no effect on cell growth. Molt 4 has an unusual phenotype in that it expresses low amounts of HLA and is expressed in conjunction with the surface antigen HTA-1 defined by the McAb NA1/34 (McMichael *et al.*, 1979). Co-expression of HLA and HTA-1 in lymphoid cells is believed to be anomalous (Bradstock *et al.*, 1980).

We show that long exposure to IFN-α leads to a dramatic increase in HLA expression resulting from substantial increases in the rate of synthesis of the specific protein. This is accompanied by a corresponding increase in the pool of mRNA containing HLA sequences. During the course of this investigation we detected a surface component in Molt 4 cells which appears after IFN-α treatment. This undescribed phenomenon was also observed in B-lymphoid cell lines.

Results

Effect of IFN-α on three surface antigens of Molt 4 cells

The effect of highly purified human IFN-α on the expression of surface membrane antigens was first investigated using the fluorescence-activated cell sorter (FACS). Three different cell surface markers expressed by Molt 4 cells were analysed using McAb W6/32 for the detection of HLA, A,B, and C antigens (Barnstable *et al.*, 1978), NA1/34 for the human thymocyte marker HTA-1 (McMichael *et al.*, 1979), and YD1/23 to recognise a leucocyte antigen (Janossy *et al.*, 1980). These three McAbs bind to 100% of the Molt 4 cells. The fluorescence profiles obtained with cells treated with IFN-α for 36 h are shown in Figure 1. The anti-HLA associated fluorescence (W6/32) increased in the IFN-α-treated cells, while neither of the two different antigens recognised by NA1/34 and YD1/23 on the surface of the same cells were affected by the IFN-α treatment. No difference in the size or shape of the cells was detected by the scatter-profiles of untreated and IFN-α-treated cells.

A time-course of IFN-α treatment shows that a lag of ~10 h is required to detect a change in the fluorescence profile. Longer periods of incubation produce a continuous increase in the expression of HLA on the membrane of Molt 4 cells, reaching a maximum at about day six (Figure 2). The relative fluorescence intensity values indicate that the long-term treated cells express as much as 8–10 times the amount of HLA as the control cells. An examination of the shape of the fluorescent profile and the shift of its position suggests that the increase in HLA takes place in a somewhat synchronised way over the totality of the cells. We did not detect inhibition of growth of Molt 4 cells after long-term exposure with IFN-α at the concentrations used (up to 10 000 units/ml). Removal of IFN-α after short- or long-term exposure (up to 20 days) resulted in a complete reversal of the effect. This, however, was a slow process, taking several days to reach the original level of expression (results not shown).

The increase in the binding of fluorescent antibody is due to an increase in the number of HLA molecules on the membrane. This was demonstrated by iodination of membrane proteins from untreated and IFN-α-treated cells and specific immuno-adsorption of the cell lysates with Sepharose-coupled McAb W6/32 and NA1/34. The eluted materials

[1]HLA is used as HLA-A,B,C unless otherwise stated.
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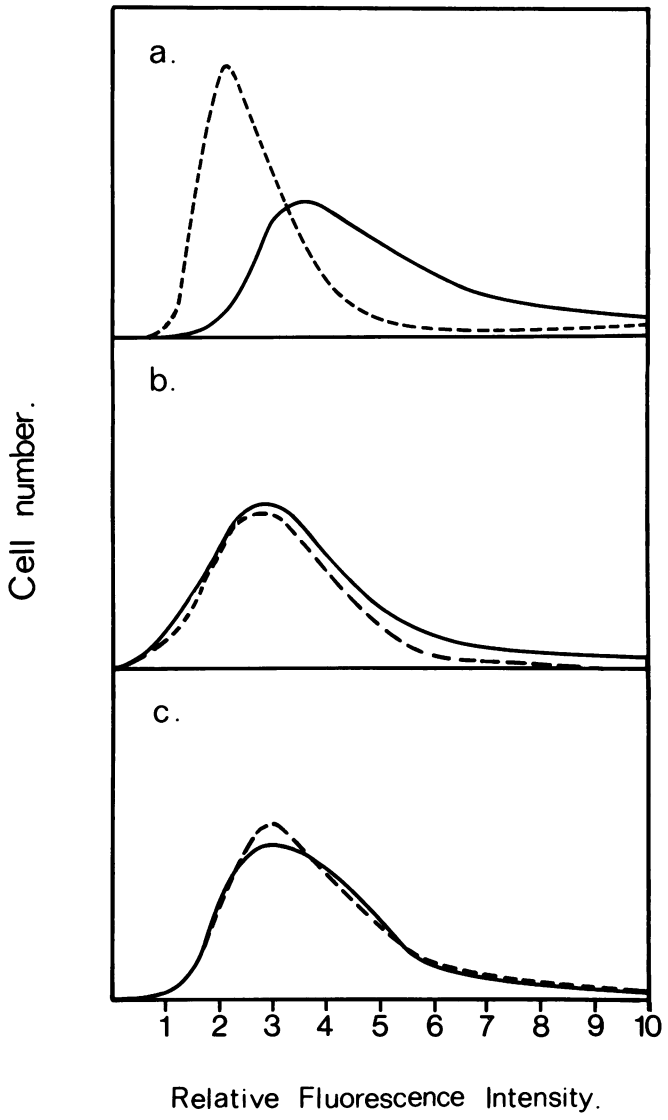

Fig. 1. Effect of IFN-α on cytofluorimetric profiles of Molt 4 cells. Untreated (-------) or IFN-α-treated cells (———) were reacted with the McAb W6/32 (a), NA1/34 (b), and YD1/23 (c) followed by fluorescein conjugated rabbit anti-mouse IgG. IFN-α treatment was for 36 h at 2000 U/ml.

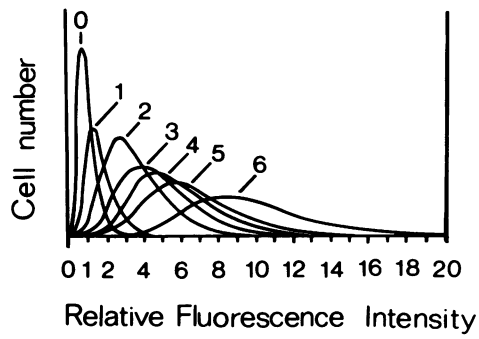

Fig. 2. Kinetics of IFN-α induced increase of HLA expression. Molt 4 cells were incubated with IFN-α at a concentration of 2000 U/ml for different times. Cells were labelled with the McAb W6/32 and fluorescein conjugated-second antibody and analysed in the FACS. All profiles were obtained the same day and are shown superimposed. The number above each profile is the number of days of IFN-α exposure.

were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), as shown in Figure 3. A clear difference in the radioactivity corresponding to the HLA heavy chain between the IFN-α-treated and untreated cell was obtained (Figure 3a). An increase in the β2m was also visible. On the other hand, the NA1/34-purified material remained unchanged, thus providing an internal control. In a different experiment, a mixture of the two Sepharose-coupled antibodies (W6/32 and NA1/34) was used to immuno-adsorb both antigens from the same aliquot of iodinated cell lysates, and the HLA increase was measured by scanning densitometry (Figure 3b). In similar experiments we have also observed that IFN-α treatment of the B-cell line RAJI specifically increases HLA (A,B,C) antigens but not HLA-DR antigens (data not shown).

Effect of IFN-α on the synthesis of HLA and β2m

The observed increase in the number of HLA molecules on the surface of IFN-α-treated cells could be due to a specific increase in the rate of synthesis and deposition on the membrane, or to a decrease in the turnover of the membrane protein. Consequently, untreated and IFN-α-treated cells were pulse-labelled with [^{35}S]methionine for 3 h, lysed and the lysates used for immunoprecipitation with W6/32 and with rabbit anti-human β2m serum. The immunoprecipitates were then analysed on a SDS-PAGE as shown in Figure 4. Cells grown in the presence of IFN-α for a short (20 h, Figure 4a) or a long (14 days, Figure 4b) period show a significant increase in the amount of newly synthesised HLA and β2m molecules. Although the results obtained with the two antibodies (W6/32 and anti-β2m serum) are very similar, the amount of the HLA chain precipitated with W6/32 from untreated cells (but not from IFN-α-treated ones) is lower than with anti-β2m serum. As expected, anti-β2m precipitates more β2m than W6/32. The presence in Molt 4 of β2m not associated with HLA as recognised by W6/32 has been reported previously (Tada *et al.*, 1978; Ziegler and Milstein, 1979). In addition, the secretion of free, newly synthesised β2m was also found to be increased by the IFN-α treatment (data not shown).

All these results demonstrate that it is the rate of synthesis of HLA and β2m molecules that has been affected by the IFN-α treatment, thus leading to the enhanced expression of the antigens on the membrane. This increased rate of HLA synthesis is due to an increased pool of HLA mRNA. This was demonstrated by a "Northern" blot analysis as shown in Figure 5. For this analysis the poly(A)-containing RNA from control and IFN-α-treated Molt 4 cells was subjected to agarose gel electrophoresis and transferred to nitrocellulose paper. The paper was hybridised with [^{32}P]DNA from clone p001 which contains sequences coding for HLA-B. A very substantial increase in the amount of mRNA present in the interferon treated cells was observed. The experiments shown in Figure 5 gave an estimated enrichment of mRNA of ~10–20 times. Therefore, the increased rate of synthesis of HLA molecules is correlated with an increased concentration of mRNA containing HLA coding sequences.

The induction of expression of p16 surface protein

Analyis by SDS-PAGE of iodinated surface proteins revealed the presence of a band (mol. wt. 16 000) observed only after IFN-α treatment. We have detected this same band in all three human cell lines tested: Molt 4, RAJI, and DAUDI (Figure 6). The p16 band was prominent and did not require antibody precipitation to reveal it. Indeed, it was one

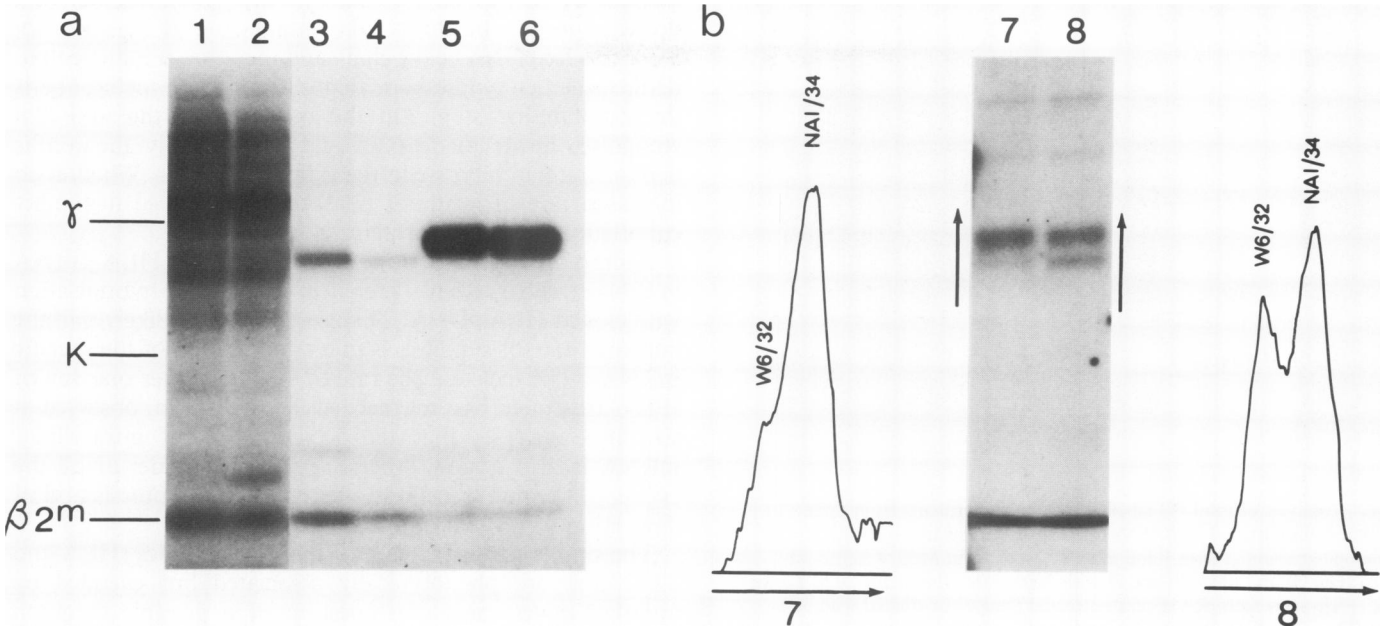

Fig. 3. Analysis by SDS-PAGE of ^{125}I-labelled surface proteins from Molt 4 cells following treatment with IFN-α. Untreated (**lanes 1, 4, 5, 7**); IFN-α-treated for 20 h with 2000 U/ml (**lanes 2, 3, 6, 8**). (**a**) Total lysates (**lanes 1** and **2**) were immuno-adsorbed with Sepharose-coupled McAb W6/32 (**lanes 3** and **4**) and NA1/34 (**lanes 5** and **6**). (**b**) Lysates from non-interferon (**lane 7**) and IFN-α-treated cells (**lane 8**) were immuno-adsorbed on a column containing both Sepharose-coupled McAb W6/32 and NA1/34. The arrows indicate the directions of the scanning densitometry of these two lanes (**7** and **8**) shown at each side. Mol. wt. markers of mouse IgG polypeptides γ (~50 000) and κ (~25 000) are indicated. The gels were 15% acrylamide.

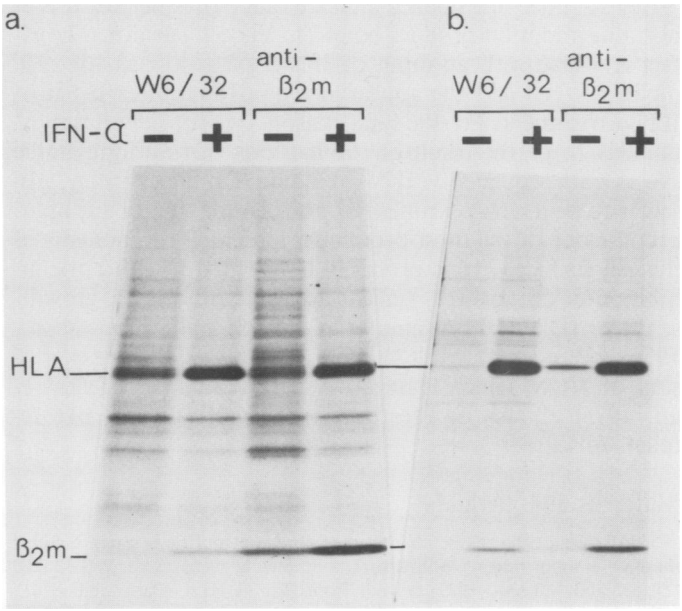

Fig. 4. Analysis by SDS-PAGE of *in vivo* [^{35}S]methionine-labelled HLA antigens from Molt 4 cells after treatment with IFN-α. Cells were either treated or not treated with IFN-α (2000 U/ml) for 20 h (**a**) or 14 days (**b**), and then pulse-labelled with [^{35}S]methionine for 3 h. Cell lysates were immuno-adsorbed with W6/32-Sepharose or immunoprecipitated with anti-β2m serum. The gels were a gradient of 8–20% acrylamide.

of the major iodinated components of the membrane proteins.

The amount of IFN-α added in the experiments shown in Figure 6 varied in the different lines from 2000 units/ml in Molt 4 to 500 units/ml in RAJI and 100 units/ml in DAUDI. This 20-fold variation did not significantly affect the intensity of the band. The mobility of p16 in SDS-PAGE was different from that of iodinated input IFN-α. In addition, p16 was not

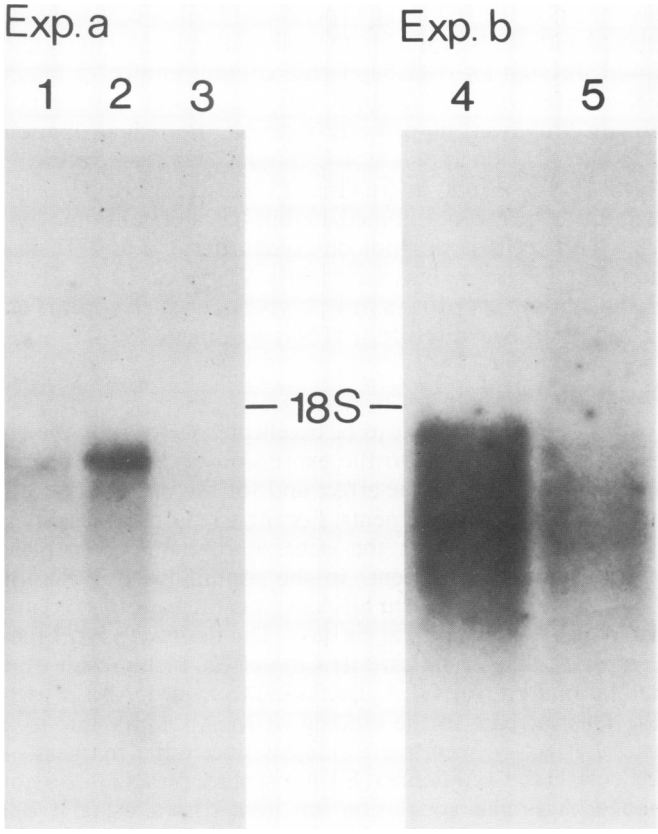

Fig. 5. HLA-related mRNA sequences present in Molt 4 cells. Total cytoplasmic mRNA from untreated and IFN-α-treated (2000 U/ml, 10 days) Molt 4 cells were electrophoresed on an agarose gel, blotted onto nitrocellulose paper and hybridised to [^{32}P]DNA from a clone containing HLA-B sequences. Experiment a, **lanes 1** and **2**: 0.7 μg and 2 μg mRNA from IFN-α-treated cells; **lane 3**: 2 μg mRNA from untreated cells. Experiment b, **lane 4**: 2 μg mRNA from IFN-α-treated cells; **lane 5**: 10 μg mRNA from untreated cells.

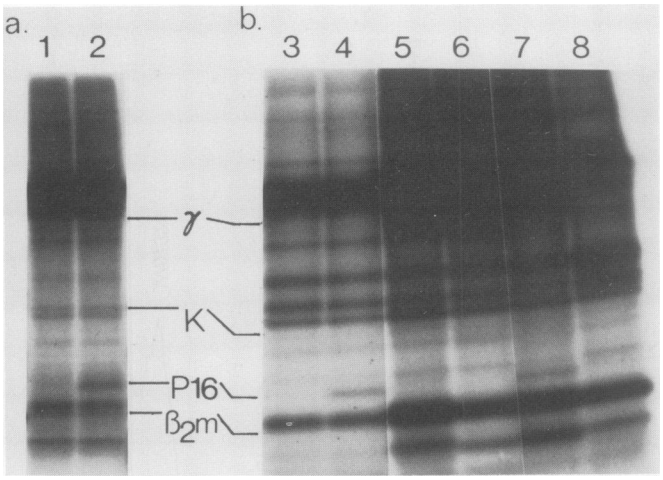

Fig. 6. Analysis of total ^{125}I-labelled cell surface proteins from three human cell lines treated with IFN-α. SDS-PAGE was in 8–20% acrylamide gradient. **Lanes 1** and **2**, [^{125}I]lysates from Molt 4 cells, untreated (**lane 1**) and treated (**lane 2**) with 2000 U/ml IFN-α for 20 h. **Lanes 3–6**, [^{125}I]lysates from RAJI cells untreated (**lane 3**) or treated with 500 U/ml IFN-α for 20 h (**lane 4**), 1 h at 4°C (**lane 5**) and 1 h at 37°C (**lane 6**). **Lanes 7–8**, [^{125}I]lysates from DAUDI cells, untreated (**lane 8**) or treated (**lane 7**) with 100 U/ml IFN-α for 20 h. Mol. wt. markers correspond to mouse IgG γ (~50 000) and κ (~25 000) chains, and human β2m (~11 500).

recognised by the NK2 McAb used to purify the IFN-α. This was shown in the following experiment. Molt 4 cells were incubated with IFN-α for 17 h, the cells iodinated and the lysate passed through an NK2-Sepharose column known to retain IFN-α. p16 was not retained by the column (results not shown). Moreover, no p16 was observed after 1 h incubation of RAJI cells with IFN-α at 4°C (Figure 5), a temperature at which IFN-α is not internalised (Stewart, 1979). The appearance of p16 is not immediate but has a certain time lag. With RAJI cells it was not observed after 1 h at 37°C, as shown in Figure 5, but was clearly visible at 12 h. These results suggest that p16 is a new polypeptide which appears on the cell surface as a result of IFN-α treatment.

Discussion

Molt 4 cells turned out to be excellent cells to study the effect of human IFN-α on the expression of HLA antigens, both for the extent of the effect and for the possibility it offers for long-term experiments. For these reasons it was possible to demonstrate that the effect is reversible even after weeks of IFN-α treatment. In the continuous presence of IFN-α, a steady increase in HLA expression was observed until a plateau was reached at six days. The fluorescence profiles (Figures 1a and 2) indicate that the effect is the result of a gradual build-up of HLA, accessible to the McAb, in each cell. This increase in the binding of McAb is not due to a general effect on membrane antigens since other markers – particularly the well-defined HTA-1 surface protein – do not change. A similar conclusion has been drawn using B cell lines and peripheral B and T lymphocytes (Fellous *et al.*, 1979, 1981; Heron *et al.*, 1978). More importantly, a direct increase in the amount of HLA and β2m chains was demonstrated by SDS-PAGE analysis of the surface-iodinated proteins.

The pulse-labelling experiments of Figure 4 demonstrate that the build-up of HLA surface protein is the result of an increase in the rate of synthesis and deposition and not of a decrease in the turnover. The antibody W6/32 does not recognise free HLA heavy chain and therefore it is difficult to define whether synthesis or deposition on the membrane is the rate-limiting step. On the other hand, the anti-β2m recognises newly made β2m and consequently the results demonstrate an increase in the rate of synthesis. Whether an increased rate of synthesis is all that is involved in the accumulation of surface proteins is uncertain.

A dramatic increase in the concentration of HLA mRNA in IFN-α-treated cells has been demonstrated by hybridisation with cloned HLA cDNA. Therefore, it is most likely that the increased rate of synthesis is due to a build-up of the specific mRNAs. The fact that the rate of synthesis after one day of IFN-α treatment has not reached the maximum, observed at six days, suggests that the accumulation of intracellular mRNA is also a slow process. A precise model of all the events will require kinetic studies of mRNA synthesis and of deposition of protein on the membrane.

The simultaneous increase of two polypeptides (HLA and β2m) that are phenotypically associated but encoded by unlinked genes raises the question of a possible common mechanism of control of quantitative expression. Other mRNAs and proteins have been shown to be enhanced by IFN-α treatment in mouse tumor cells and in HeLa cells (Farrell *et al.*, 1979; Gupta *et al.*, 1979; Broeze *et al.*, 1980). Here we report the induction of expression of a surface molecule, p16, that appears to be common to B and T lymphoid lines.

Several lines of evidence indicate that p16 is neither IFN-α nor a degradation product bound to the cell surface. The effect is visible with an input of IFN-α of as low as 10^4–10^5 molecules/cell and the appearance of p16 requires >1 h of IFN-α treatment. For the same reasons, we consider it is most unlikely to be the result of "unmasking" of an iodinatable site as a consequence of IFN-α binding. On the contrary, p16 seems to be a newly synthesised polypeptide that builds up to become one of the most prominent iodinated components of the cell surface. p16 is probably not related to either the inhibition of cell growth, since it appears on both cell growth-sensitive (DAUDI, Adams *et al.*, 1975; Hilfenhaus *et al.*, 1976) and resistant (Molt 4) lines, or to the cell surface expression of HLA, which is negative in DAUDI (Goodfellow *et al.*, 1975). Otherwise the functional significance of p16 remains obscure.

Materials and methods

McAbs were either tissue culture supernatants or purified from the ascitic fluid of tumour-bearing animals. The following were used: W6/32 HL (Ziegler and Milstein, 1979) derived from W6/32 HLK (Barnstable *et al.*, 1978), NA1/34 (McMichael *et al.*, 1979) and YD1/23 (Ziegler and Milstein, unpublished; Janossy *et al.*, 1980).

Immuno-adsorbants were prepared with Pharmacia CNBr-activated Sepharose 4B; protein-A Sepharose CL-4B and poly(U)-Sepharose were from Pharmacia. Rabbit anti-human β2m was a kind gift from M.Owen.

NK2-IFN-α was kindly provided by D.S.Secher. It was a highly purified form of specific activity >2 x 10^8 U/mg, obtained by purification with the anti-IFN-α McAb NK2 (Secher and Burke, 1980), of crude interferon kindly provided by K.Cantell.

Cytofluorimetric analysis

Indirect fluorescence staining was performed with 0.25 ml/10^7 cells of culture supernatants of the McAb. The developing antibody was fluorescein isothiocyanate (FITC)-labelled rabbit anti-mouse (or anti-rat) IgG (Miles) made up in Dulbecco's phosphate buffered saline (DPBS)-10% foetal calf serum (FCS). Both incubations were for 60 min at 4°C. After each treatment cells were washed twice with DPBS-10% FCS and finally resuspended in the same buffer (10^7 cell/ml) for the quantitative analysis in the FACS II.

Cell surface iodination

Lactoperoxidase catalysed iodination was performed by incubation of 10^7 cells in 1 ml of PBS containing 1 mCi ^{125}I (Amersham, 13–17 mCi/μg), 2.5 μM KI, 200 mg/ml lactoperoxidase, and 0.006% H_2O_2 for 10 min at room temperature. The reaction was stopped by addition of 5 μl tyrosine-saturated solution and the cells washed three times with PBS at 4°C. Cells were finally lysed in PBS containing 0.5% NP40 and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min at 4°C, nuclei removed by centrifugation, and the supernatant dialysed overnight against PBS.

Analysis of biosynthetically labelled proteins

[^{35}S]Methionine incorporation was done by incubating cells at 5–6 x 10^6 cells/ml in methionine-free medium containing 150 μCi/ml [^{35}S]methionine (Amersham, 1410 Ci/mmol). Cell lysates were obtained by resuspending the cells in 0.5% NP40 made up in 100 mM Tris-HCl pH 8, 250 mM NaCl 1 mM PMSF buffer for 30 min at 4°C, spinning at 2000 r.p.m., and taking the supernatant.

Immuno-adsorption was performed on 30–60 μl of beads of Sepharose-coupled antibody for 2–3 h at 4°C. Beads were washed by passing sequentially 20 volumes of each of the three following solutions: (a) 0.1 M Tris, pH 8, 0.25 M NaCl, 0.5% NP40, 1 mM PMSF, 1 mg/ml bovine serum albumin (BSA); (b) 50% (v/v) ethylene glycol, 30% (v/v) PBS, 0.3 M NaCl, 1 M PMSF, 0.5% NP40; and (c) as (a) but without albumin. Finally the material bound was eluted with 0.1 M glycine-HCl pH 2.5 and neutralised with 1 mM Tris pH 8. SDS-PAGE was performed according to Laemmli (1970). 8–20% acrylamide linear gradients were made using the same buffers.

For ^{35}S detection gels were fluorographed as described by Laskey and Mills (1977).

Analysis of mRNA and hybridisation

Poly(A)-containing mRNA was obtained from total cytoplasmic RNA by purification on poly(U)-Sepharose chromatography. Glyoxal denaturation of mRNA, agarose electrophoresis, and blotting onto nitrocellulose paper (Schleicher and Schuell) were performed as described by Thomas (1980). The RNA on the filters was hybridised with a radioactive human HLA-B cDNA clone kindly provided by S.M.Weissman (clone p001, Sood *et al.*, 1981). The DNA probe was nick-translated to a specific activity of 10^8 d.p.m./μg with [^{32}P]dATP and [^{32}P]dCTP (Amersham) and hybridised for 60 h at 42°C in 50% (v/v) formamide, 5 x SSC (0.75 M NaCl/0.075 M trisodium citrate) 0.2% each BSA, Ficoll and polyvinylpyrrolidone, 0.1% SDS, and 50 μg/ml of sonicated denatured salmon sperm DNA. Results were visualised by autoradiography of the filters.

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