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Stimulation of HLA-A,B,C by IFN-α. The derivation of Molt 4 variants and the differential expression of HLA-A,B,C subsets

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Spontaneous mutants with altered HLA-A,B,C response to interferon-α (IFN-α) were isolated from the human thymus leukemia cell line Molt 4. Using fluorescein isothiocyanate (FITC)-conjugated W6/32 (a monoclonal antibody to HLA-A,B,C) and the fluorescence-activated cell sorter, the cells with highest and lowest fluorescence after 24-48 h of IFN-α treatment were selected and expanded. After several cycles of selection, mutant clones with low (>10% of wild-type) and high (three times better) response were obtained. A similar protocol was employed to derive high responder mutants with the monoclonal antibody YT76, which recognises a subset of HLA strongly induced by IFN-α. Stable clones were derived for which YT-HLA induction was 7-fold that of Molt 4 cells and for which HLA induction occurred at 100-fold lower concentrations of IFN-α. The high response phenotype of the mutants was not accompanied by a significant increase in the constitutive level of expression of HLA-A,B,C (in the absence of IFN). The increase in the level of HLA-A,B,C expression after IFN-α treatment is mostly accounted for by the increase in the expression of a subset of HLA molecules, detected by the monoclonal antibody YT76 including HLA-B molecules.
Key words: control of expression/HLA/interferon/somatic mutation

Introduction

The interferons (IFNs) are a group of closely related naturally occurring proteins with potent anti-viral and growth-inhibitory effects (for review see Lengyel, 1982). In addition they induce the production of several cell surface proteins (Fellous *et al.*, 1979; Chebath *et al.*, 1983; Wallach, 1983), including the human transplantation antigens HLA-A,B,C (Heron *et al.*, 1978; Fellous *et al.*, 1981; Burrone and Milstein, 1982a). Since HLA-A,B,C serve to restrict and focus the activity of cytotoxic T lymphocytes against virus-infected and tumour cells (reviewed in Nabholz and MacDonald, 1983) the regulation of HLA expression by the IFNs may have major immunoregulatory implications. To clarify further this effect we sought to derive mutants of the IFN-induced HLA response (Burrone and Milstein, 1982b).

The expression of the HLA-A,B,C antigens is almost ubiquitous in human tissues (McMichael, 1982). Yet not only are they subject to quantitative changes of expression following IFN treatment in lymphoid cells and cell lines from other tissues (Burrone and Milstein, 1982a; Fellous *et al.*, 1982; Basham *et al.*, 1982), but this effect is particularly noticeable when the constitutive level of expression is weak, as, for example, in cortical thymocytes (McMichael *et al.*, 1979; Diaz Espada, Secher and Milstein, unpublished). Molt 4 is a thymus-derived cell line which constitutively expresses small amounts of HLA-A,B,C but which, after exposure to IFN-α, expresses high levels of these cell surface proteins with a corresponding increase in expression of HLA mRNA (Burrone and Milstein, 1982a). It is therefore an attractive model for the study of the regulation of HLA expression by IFN.

We have used the monoclonal antibody (McAb) W6/32, which detects all HLA-A,B,C (Barnstable *et al.*, 1978), and the McAb YTH/76.3 (for simplicity we refer to it as YT76), which recognises a subset of HLA-A,B,C molecules (Clark *et al.*, 1985), to sort and clone Molt 4 cells which express high or low levels of HLA on induction with IFN-α. We have thus derived a series of stable mutant cell lines, whose varying response and sensitivity to IFN may facilitate a closer analysis of HLA regulation.

Results

Selection of IFN-response variants with W6/32

Molt 4 cells were treated for 24 h with 2000 U/ml IFN-α, conditions under which the expression of HLA-A,B,C molecules on the cell surface increase ~2-fold. Cells were labelled with FITC-W6/32, a McAb recognising HLA-A,B,C molecules, and two fractions containing the cells with high and low fluorescence intensity values were sorted using fluorescence-activated cell sorter (FACS) (Figure 1). Each fraction containing 3-5% of the original population was independently grown for ~10 cell generations in the absence of IFN and then submitted to a new cycle of IFN treatment and sorting.

To assess the progress of the selection at different stages, the HLA-A,B,C content of untreated or IFN-α-treated cells was

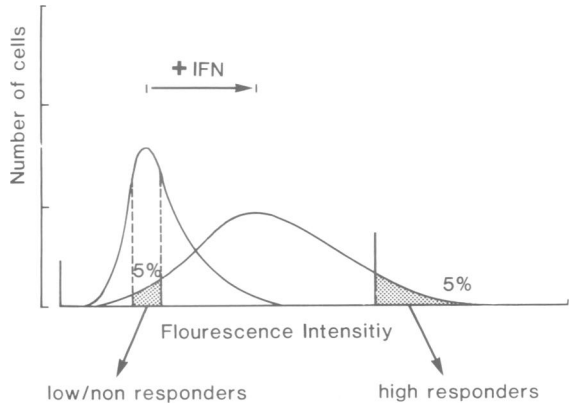

Fig. 1. Strategy for the selection of IFN-induced HLA-A,B,C responder mutants. Cytofluorometric profiles of Molt 4 cells, untreated or treated with IFN-α for 24 h, are shown schematically. The shadowed areas indicate the fractions of cells sorted on each cycle during the selection of low/non-responder and high responder mutants.

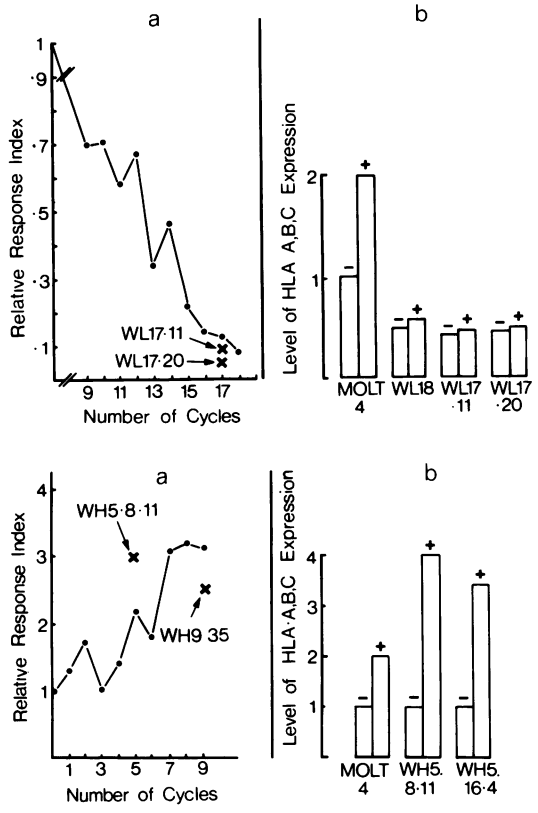

Fig. 2. Response phenotype during the selection of 'WL' low/non-responder (top) and 'WH' high responder (bottom) mutants with W6/32. HLA-A,B,C levels of expression were determined by cytofluorometry of cells labelled with FITC-W6/32. (**a**) The response index was defined as the increase in fluorescence induced by IFN (2000 U/ml, 24 h) divided by the fluorescence in the absence of IFN. Values were expressed relative to Molt 4, whose response index was taken as 1. (x) represents isolated clones. (**b**) Levels of HLA-A,B,C surface expression in non-treated (−) and IFN-treated (+) wild-type Molt 4 cells and mutants were expressed relative to the constitutive level (absence of IFN) of HLA-A,B,C in Molt 4.

determined by quantitative cytofluorimetry using FITC-conjugated W6/32. The increase in fluorescence due to IFN, divided by the fluorescence in its absence, is referred to as IFN-induced HLA-response.

Figure 2 (top) shows the phenotypic characteristics obtained during the selection with W6/32 of low/non-responder populations, expressed as the IFN-induced HLA-response relative to the wild-type Molt 4. A significant alteration in the response was first detected at cycle No. 9. At this stage, the IFN treatment was increased to 48 h, thus allowing a better separation of the low/non-responsive cells. Cycle 18 marked the last round of selection, and the cells obtained, W6/32-Low ('WL') 18 showed a clear low responder phenotype (8% response compared with Molt 4). Twelve clones were isolated from the population at stage 17 and they all showed a very low response phenotype of between 6% and 15% relative to Molt 4. Panel b shows the level of expression of HLA-A,B,C antigens in the wild-type and mutants, including clones WL17.11 and WL17.20, in the absence and presence of IFN. Apart from their low response to IFN-α, all 12 clones as well as the uncloned cells, have a constitutive level of expression of HLA-A,B,C antigens of about one half of the wild-type. It should be noted that this low constitutive level was obtained before any significant change in IFN response was observed.

The evolution of the selection with W6/32 of high responder

Table I. Response to IFN-α of HLA expression variants

Cells	McAb	Relative fluorescence		Response[a]	
		No IFN	IFN	Index	Relative to M4
Experiment 1			2000 U/ml		
Molt 4	W6/32	56	188	2.4	1.0
WL		17	22	0.3	0.04
WH		63	520	7.3	3.5
YH		60	368	5.1	2.3
Molt 4	YT76	16	116	6.3	1.0
WL		12	18	0.5	0.06
WH		16	540	33.0	5.2
YH		20	680	33.0	6.4
Experiment 2			100 U/ml		
Molt 4	W6/32	50	72	0.5	1.0
YHH		55	384	6.0	15.0
YHHH		58	504	7.7	20.3
Molt 4	YT76	13	26	1.0	1.0
YHH		16	248	14.5	17.8
YHHH		22	624	27.3	46.3

Cells were exposed to IFN-α for 48 h at the concentrations shown and the relative fluorescence after staining with McAb determined as described in Materials and methods.
[a]The Response Index is defined as (IFN induced − non-induced fluorescence/non-induced fluorescence), and the Response Relative to M4 as IFN induced-non induced fluorescence in Molt 4/ in individual line. WH is the clone WH5.8.11, YH is the clone YH5.18, YHH is the clone YH5.18H7.35.11 and YHHH is the line YH5.H7.H7.

cells (W6/32-High = 'WH') is shown in Figure 2 (bottom). The presence of a high responder variant was clearly seen as a second population after the fifth cycle (not shown). At this stage cells were cloned in soft agar. About 40% of them had a high-response phenotype, some of them better than average (e.g., WH5.8.11). A second set of clones was later derived after the 9th round of selection (WH9 clones). Clones WH5.8.11 and WH9.35 have an IFN-induced HLA response three times greater than the wild-type Molt 4. In other words the number of HLA molecules on the membrane, increases by a factor of four after 24 h of treatment with IFN-α, whereas Molt 4 HLA barely doubles under the same conditions (Figure 2). While the IFN-dependent HLA expression is higher in the mutant cells than in the wild-type Molt 4, the basal constitutive levels remain unchanged (Table I). This is true for all clones tested, and it is a clear indication of the regulatory nature of these new mutant cell lines. The enhanced response to IFN is not due to changes in the kinetics of the effect, since similar results are obtained at all different times of IFN treatment (Burrone and Milstein, 1982b). For instance, clone WH5.16.4 increases the amount of membrane HLA-A,B,C > 20 times after 5 days of exposure to IFN.

We have previously shown that IFN-α increases the HLA-A,B,C expression in Molt 4 by increasing the relative concentration of mRNA molecules coding for the specific antigens (Burrone and Milstein, 1982a). Similar studies were performed on the mutants described above. Figure 3 shows the result of an RNA blot analysis performed on poly(A)-containing RNA derived from both IFN-treated and untreated wild-type Molt 4 and the high-responder mutant WH5.8.11. The results show a significantly larger increase in the HLA mRNA concentration in WH5.8.11 cells after 24 h of IFN treatment, compared with Molt 4 cells treated for 6 days.

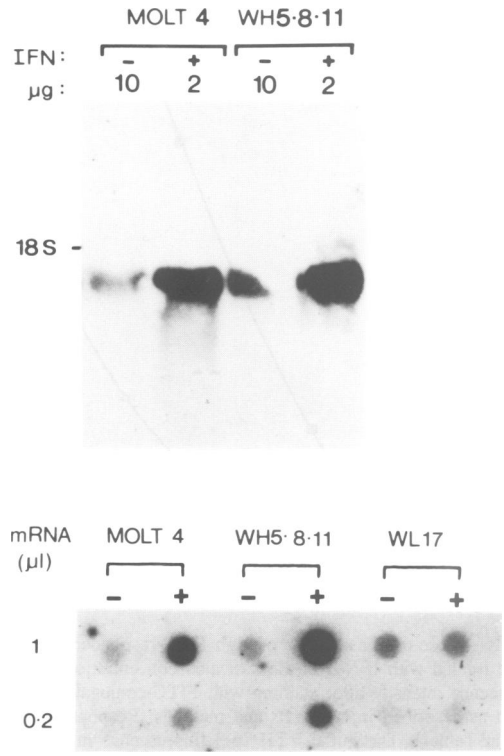

Fig. 3. HLA-mRNA sequences in wild-type Molt 4 and IFN-α responder mutants. Total poly(A)-containing RNA was either (**a**) electrophoresed on agarose gel and transferred, or (**b**) directly blotted onto a nitrocellulose filter. mRNA was derived from non-treated (−) and IFN-treated (+) cells. Molt 4 cells were treated with IFN-α for either 6 days (top) or 24 h (bottom); while WH5.8.11 and WL17 cells were treated for 24 h. Filters were hybridized to a [^{32}P]DNA from an HLA-B cDNA clone and then subjected to autoradiography.

We also performed dot-blot hybridization assays using poly(A)-containing RNA (Figure 3) and total cytoplasmic extracts (not shown). The phenotype of the mutants correlates with the capacity of IFN to induce an increase in HLA mRNA levels. This was true for both high and low responder clones.

Selection of variants with YT76

In separate sorting experiments we chose to select variant clones of Molt 4 with the McAb YT76. In contrast to W6/32, which recognises all HLA-A,B,C molecules, YT76 recognises a sub-population of HLA-A,B,C molecules. As Figure 4 shows, YT76 immunoprecipitates a radioiodinated cell surface dimer of mol. wt. 45 k and 12 k. Pre-clearing a cell membrane lysate by exhaustive immunoprecipitation with the pan-HLA reacting McAb W6/32 removes all YT76-reactive material, but not vice versa (Figure 4). YT76 was chosen as an agent in the selection of IFN-α response variants because Molt 4 cells were very weak or negative for YT76 fluoresence, but strongly positive after stimulation with IFN (Table I). YTH/76 therefore appeared to recognise an IFN-induced subpopulation among the HLA-A,B,C antigens, probably products of the B locus (Clark *et al.*, 1985).

The evolution of the selection with YT76 of high responder cells after stimulation for 24 h with 2000 U/ml IFN-α was similar to that described above for the WH variants. The presence of the high responder population was clearly visible at the fifth cycle of selection, at which stage the cells were cloned. The clone YT-High ('YH')5.18 has a YT76 response to this concentration of IFN which is 6-fold higher than the response of wild-type Molt 4 cells (Table I). On the other hand, the increment in W6/32 fluorescence reflecting the overall increase in HLA-A,B,C, is in YH cells a little lower than that of the variant WH (Table I). The induction of HLA-A,B,C by IFN as detected by W6/32 can largely be accounted for by the increase in that subpopulation of HLA detected by YT76 whether the analysis is performed by

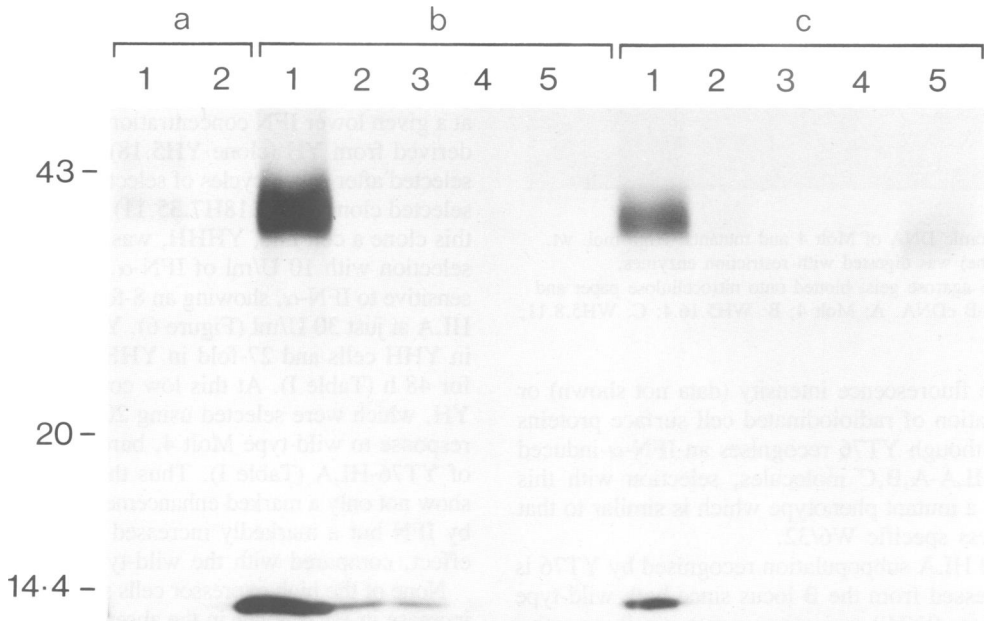

Fig. 4. Sequential immunoprecipitation with W6/32 and YT76 of radioiodinated cell surface proteins from IFN-α-induced and non-induced high expressor variants of Molt 4. (**a**) An equal aliquot of radioiodinated cell surface proteins from non-IFN treated YHHH cells was immunoprecipitated with W6/32-Sepharose (**lane 1**) and YT76-Sepharose (**lane 2**). (**b**) Labelled cell surface proteins from YHHH cells stimulated 48 h with 1000 U/ml IFN-α were immunoprecipitated with W6/32-Sepharose (**lane 1**) as described in Materials and methods, and the procedure repeated until all W6/32-detected antigen was cleared from the lysate (**lanes 2–4**). Final immunoprecipitation was with YT76 (**lane 5**). (**c**) An equivalent aliquot of the same lysate was immunoprecipitated with YT76-Sepharose (**lane 1**) until all detected antigen was cleared (**lane 2–4**), before a final immunoprecipitation with W6/32-Sepharose (**lane 5**).

Table II. HLA serotypes of IFN-response variants

Cells	No IFN	IFN
Molt 4 (Brodsky *et al.*, 1979)	A1/A10	
Molt 4	?A1 CW2?	A1, A10, B17?
WH	?A1	A1, A10, B17, B18, CW2
YH	–	A1, A10, B17, B18?, CW2?
YHH	–	A1, A10 (A25)[a], B17, B18, CW2

Cells, untreated or treated for 48 h with 2000 U/ml IFN-α, were kindly serotyped by Dr D.Voak at the Rgional Transfusion and Immuno-Haematology Centre, Long Rd., Cambridge. WH is WH5.8.11; YH is YH5.18 and YHH is YH5.18H7.35.11.
[a]A25 is one of the two specificities in which A10 splits.

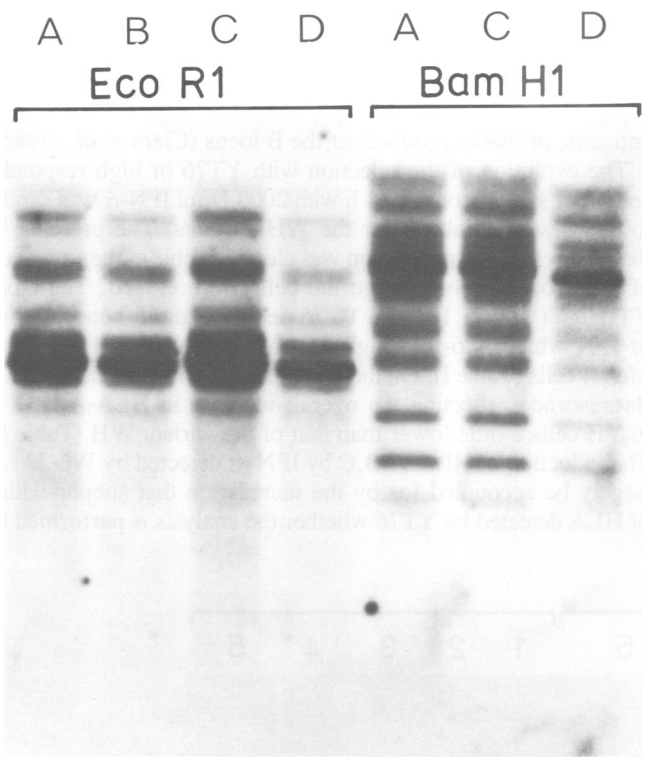

Fig. 5. Analysis of genomic DNA of Molt 4 and mutants. High mol. wt. DNA (12 μg in each lane) was digested with restriction enzymes, electrophoresed in 0.7% agarose gels, blotted onto nitrocellulose paper and hybridized to [^{32}P]HLA-B cDNA. **A**: Molt 4; **B**: WH5.16.4; **C**: WH5.8.11; **D**: WL17.

relative cell-surface fluorescence intensity (data not shown) or by immunoprecipitation of radioiodinated cell surface proteins (Figure 4). Thus although YT76 recognises an IFN-α-induced subpopulation of HLA-A,B,C molecules, selection with this McAb gives rise to a mutant phenotype which is similar to that selected with the less specific W6/32.

The IFN-induced HLA subpopulation recognised by YT76 is at least partly expressed from the B locus since both wild-type and high YT-expressor ('YH') variants are serotypically negative for HLA-B until after IFN stimulation, when Molt 4 becomes weakly B17 and B18 positive and YH strongly so (Table II). The possibility that HLA gene amplification is the genetic event underlying the phenotypic changes was investigated by Southern blot analysis of Molt 4 in the low and high responder mutants described above, using an HLA cDNA clone as a probe (Sood *et al.*, 1981). The results did not disclose any difference between the different DNAs (Figure 5).

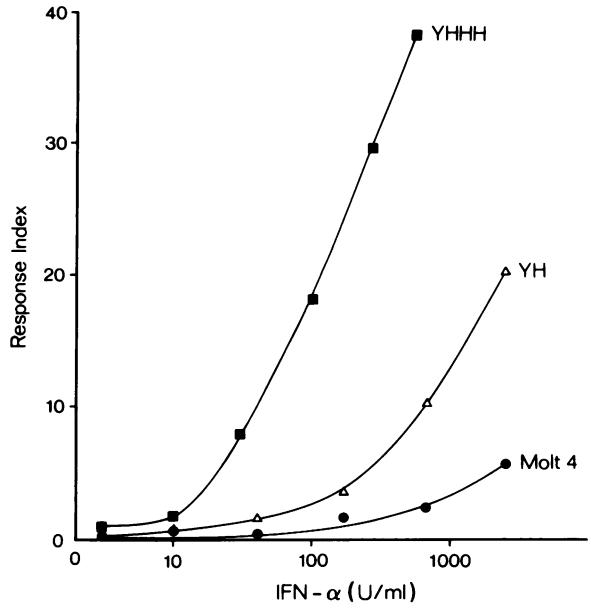

Fig. 6. IFN-α dose-response curve of high HLA-responder Molt 4 variants. Cells were treated with IFN-α at the indicated concentrations for 24 h and the fluorescence intensity after staining with FITC-conjugated YTH/76.3 determined as described in Materials and methods. Response index is as described in Table I. The initial YTH/76.3 fluorescence in non-induced cells was similar for Molt 4, YH and YHHH (see also Table I). YH is the clone YH5.18, and YHHH is the line YH5.H7.H7.

Variants responding to low concentrations of IFN-α

A further refinement in the derivation of IFN-induced HLA variants was achieved by selecting for high YT76 expression in the presence of lower concentrations of IFN-α. By successive sorting of YH cells with FITC-YT76 after 48 h incubation in successively lower concentrations of IFN-α, the cell line YHH was derived. In naming these variants we chose to suffix the letter 'H' for each line derived after a series of rounds of selection at a given lower IFN concentration. Thus the cell line YHH was derived from YH (clone YH5.18) by cloning a cell population selected after seven cycles of selection with 50 U/ml IFN-α. The selected clone (YH5.18H7.35.11) is referred to as YHH. From this clone a cell line, YHHH, was derived after seven cycles of selection with 10 U/ml of IFN-α. YHHH cells are exquisitely sensitive to IFN-α, showing an 8-fold induction of YT76-specific HLA at just 30 U/ml (Figure 6). YT76-HLA is induced 14-fold in YHH cells and 27-fold in YHHH cells by 100 U/ml IFN-α for 48 h (Table I). At this low concentration of IFN, WH and YH, which were selected using 2000 U/ml IFN, are similar in response to wild-type Molt 4, barely doubling their expression of YT76-HLA (Table I). Thus the variants YHH and YHHH show not only a marked enhancement in induction of YT76-HLA by IFN but a markedly increased sensitivity to IFN-α for this effect, compared with the wild-type parent line.

None of the high expressor cells showed significant constitutive increase in HLA levels in the absence of IFN. Furthermore, like the WH and YH lines, YHH and YHHH clones have shown a stable and reproducible response to IFN stimulation on repeated testing over a period of up to 12 months.

Discussion

The fluorescence-activated cell sorter is a powerful tool in the selection of mutants of the expression of cell surface antigens

(Neuberger and Rajewsky, 1981; Holtkamp *et al.*, 1981; Kamarck *et al.*, 1982), and we have previously described the use of this technique in the derivation of a stable cell line which, compared with wild-type Molt 4, expresses a 10-fold higher level of HTA-1 (CD 1) (Burrone *et al.*, 1983). We have extended this approach to develop mutants of regulatory functions (Burrone and Milstein, 1982b), and here we describe the derivation of several Molt 4 mutants where the IFN regulation of HLA gene expression is altered with respect to the wild-type. The procedure used did not involve mutagens and depended on the selection of spontaneously occurring variants in the wild-type population.

It is possible to make an estimate of the minimum frequency of HLA response variants in the wild-type population as the cell doubling time of wild-type and high responder variants are approximately equal. For the derivation of WH5.8.11 an average of 5% of the cells in each cycle were isolated, and the frequency of the mutant at cycle 5 was ~50%. Therefore, the mutants in the original population were present at a frequency of at least 6×10^{-6} (i.e., 0.05^4 since by the 4th cycle there must have already been between 2.5% and 5% of mutants in the population). The frequency of altered phenotype in the starting population is of a similar order to that found in various heterogeneous cultured cell lines for amplified genes (Schimke, 1984). We have not observed evidence of gene amplification of the HLA gene. Neither did we observe a high rate of reversion to low responses. Since the constitutive level of HLA-A,B,C expression in the high IFN-response variants is not significantly altered, a regulatory component may be the focus for mutation or depression. It is likely that the phenotypic alterations are the results of complex genetic and epigenetic events, especially for the YHHH cell line. Both WH and YH were easy to derive, but further sorting with 2000 units of IFN-α did not result in further improvements. When the IFN-α concentration was dropped to 50 units and later to 10 units, further improvements were possible. It is possible that the high HLA responder phenotype correlated with a growth disadvantage or an increase in sensitivity to the cytotoxic effects of IFN-α.

Of the clones isolated from the low responder population, WL, none were fully non-responder to IFN. It appears therefore that mutants altered in the quantitative expression of HLA occur at higher frequency than mutants with loss of activity. The low IFN-responder variants were more difficult to derive than high responders. In addition they gradually evolved to phenotypes with progressively lower and lower amounts of cell surface HLA and a wild-type population was never detected amongst the low responder cells. It is possible therefore that the low responders resulted from an accumulation of independent events. These could include a decrease in the number of IFN receptors (Branca and Baglioni, 1981; Aguet, 1980) perhaps associated with karyotypic changes. (Tan, 1976; Tan and Green, 1976) and successive mutations affecting regulatory sequences controlling some of the HLA-A,B,C genes.

The specificity of the McAb YT76 for an IFN-induced subpopulation of HLA-A,B,C is of special interest to study the induction by IFN, in view of the virtual absence of YT76 epitope in untreated cells, and its subsequent elevation after IFN stimulation to levels which account for nearly all the HLA-A,B,C newly expressed by the cell. The difference between the increase in expression of the W6/32 and YT epitopes indicates that the effect of IFN-α is not identical to all HLA-A,B,C molecules. The difference in the response index is due to a subpopulation of HLA-A,B,C detected by W6/32 but unrecognized by YT76. This subpopulation is obviously much less affected by IFN-α than other HLA-A,B,C components in both wild-type and mutants. It seems then that in the absence of IFN-α some of the HLA-A,B,C genes are expressed (although not necessarily fully) while others are silent. The effect of IFN-α may be to induce full activation or derepression of all of them. This increase allows detection of at least two new HLA-specificities (B17 and B18) not detected in the absence of IFN. Confirmation of the selectivity of IFN-α and a more detailed quantitation of the responses of the different HLA loci requires HLA probes derived from an IFN-induced Molt 4 cDNA library. Identification of a specifically induced HLA may be of considerable importance in understanding and modifying the immunoregulatory functions of the IFNs.

The successful derivation of high/low responder phenotype mutants by fluorescent monoclonal antibodies and FACS selection, although somewhat lengthy, yields interesting mutants. These provide important clues as to the mechanism of action of IFN in the control of HLA-A,B,C expression, and cell lines valuable for the characterization of the genes involved.

Materials and methods

Highly purified interferon (NK2-IFN-α) (Secher and Burke, 1980), a gift from Dr D.S.Secher, was prepared from crude leucocyte IFN, kindly provided by Dr K.Cantell. IFN units were calibrated with reference to the MRC 69/19 standard. The pan-HLA reacting McAb W6/32 HLK has been described previously (Barnstable *et al.*, 1978). The McAb-producing rat hybridoma line YTH/76.3 (which for simplicity will be referred to as YT76) was the kind gift of Dr H.Waldmann. McAbs were purified from the ascitic fluid of tumour-bearing mice as described by Svasti and Milstein (1972). Cytofluorimetry and cell sorting were performed using the fluorescence activated cell sorter (FACS II, Becton-Dickinson, CA). Direct immunofluorescence staining was performed using FITC-conjugated McAb prepared as described (Galfre and Milstein, 1981). For sorting, $\sim 5 \times 10^7$ cells were washed in phosphate-buffered saline (PBS)-10% foetal calf serum, and incubated with 1 ml of antibody solution (50 μg/ml) made up in the same buffer, for 1 h at 4°C. Cells were then washed twice with PBS-10% foetal calf serum and finally resuspended at 10^7 cell/ml. Sorted cells were collected in Dulbecco's modified Eagles medium-10% foetal calf serum supplemented with 10 mM Hepes buffer and later transferred to culture dishes containing a feeder layer of mouse peritoneal macrophages. Several fractions of $\sim 10^4 - 10^5$ cells were taken at each cycle and grown independently for 4–5 days, and pooled. Cell surface fluorescence was quantitated by determining the mean fluorescence intensity of $2-4 \times 10^4$ cells. Mean fluorescence intensities were calculated as the ratio Ni.Ci/Ni [Ni = number of cells in channel Ci]. Cell surface lactoperoxidase iodination and immunoprecipitations with Sepharose-bound McAb were performed as described (Burrone and Milstein, 1982a), except that the beads were washed first in 20 volumes of NNS buffer at room temperature (50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 5 mM Na_2EDTA, 0.5% NP40, 0.1% SDS), followed by 20 volumes of PBS, 0.5% NP40, 0.1% SDS, also at room temperature.

Poly(A)-containing mRNA was purified from total cytoplasmic RNA by poly(U)-Sepharose chromatography. General protocols were as previously described (Burrone and Milstein, 1982a). Total cytoplasmic lysates for dot-blot hybridizations (White and Bancroft, 1982) contained the equivalent of $5-10 \times 10^4$ cell/μl. The HLA-B cDNA DNA probe, kindly provided by Weissman (Sood *et al.*, 1981) was nick-translated to a specific activity of 10^8 c.p.m./μg with [^{32}P]dATP and [^{32}P]dGTP (Amersham). High mol. wt. DNA was digested with restriction enzymes *Eco*RI and *Bam*HI (BioLab, New England) in 60 μl reactions containing 10–15 μg DNA, and 10 times excess of the corresponding enzyme. Samples were fractionated by 0.7% agarose gel electrophoresis in 90 mM Tris-Borate (pH 8.3)-3 mM EDTA-20 mM NaOAc buffer at 1 V/cm for 15 h. The DNA on the gel was transferred onto nitrocellulose paper and hybridized to the same probe as described above for 60 h at 65°C in 0.75 M NaCl-10 mM EDTA-0.15 M Tris-HCl (pH 8.0), 0.2% each bovine serum albumin, Ficoll and polyvinylpyrrolidone, and 250 μg/ml of sonicated denatured salmon sperm DNA. Filters were washed at the same temperature in 0.15 M NaCl-2 mM EDTA-0.03 M Tris-HCl (pH 8.0)-0.1% SDS and subjected to autoradiography.

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