Int. J. Immunopharmac., Vol. 9, No. 4, pp. 459–468, 1987.
Printed in Great Britain.

0192–0561/87 $3.00+ .00

QUANTITATIVE MODIFICATIONS OF MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) ANTIGENS INDUCED BY RECOMBINANT GAMMA INTERFERON IN TWO HUMAN BREAST CANCER LINES

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(*Received* 28 *July* 1986 *and in final form* 8 *January* 1987)

Abstract — H466-B and T47-D breast carcinoma cell lines were treated with recombinant gamma interferon (rγIFN) to study major histocompatibility complex (MHC) class I and class II antigen responses. Untreated H466-B cells released B_2 microglobulin (B_2M) into the culture medium and expressed B_2M and class I heavy chain on 100% of the cells. The expression of class II antigens (DR) was limited to 8 ± 4% of the cells. This subpopulation was isolated by cell sorting and labelled with ^{35}S methionine. Protein extracts were immunoprecipitated with anti-DR antibody and subjected to two dimensional non-equilibrium pH gradient electrophoresis (2D-PAGE). A normal pattern of expression of invariant, alpha and beta chains was shown. The MHC antigenic expression of H466-B parental cell line was not modified by interferon treatment. Untreated T47-D cells did not release B_2M into the culture medium, expressed B_2M and class I heavy chain on 100% of the cells but did not express class II molecules using radio-immunoassay or 2D-PAGE. As early as 24 h after rγIFN addition, T47-D cells released B_2M into the medium, B_2M and class I heavy chain were significantly greater than that of untreated cells, and class II antigenic expression was found, all these in a dose dependent manner. 2D-PAGE analysis of class II antigens revealed the profile of human DR molecules but this expression seemed incomplete since only single alpha and beta spots were detected suggesting a possible defect in the sialilation of DR molecules.

These results show a heterogeneity in MHC antigenic responses to rγIFN and suggest that synthetized class II molecules may be incompletely processed.

Interferons exert a large variety of biological effects on cells (Bloom, 1980) among which many may have wide implications for an antineoplastic activity such as the modulation of MHC gene product expression (Dolei, Capobianchi & Ameglio, 1983; Rosa & Fellous, 1984) and the induction of malignant cell differentiation (Hattori, Pack, Bougnoux, Zong Liang Chang & Hoffman, 1983). The effects of interferons on MHC antigens of human sarcoma and carcinoma cells have been well documented. They have been shown to be heterogenous (Carrel, Schmidt-Kessen & Giuffre, 1985; Pfizenmaier, Bartsch, Scheurich, Seliger, Ucer, Vehmeyer & Nagel, 1985; Schwartz, Momburg, Moldenhauer, Dorken & Schirrmacher, 1985) and seemed to be related not to the absence of interferon receptors on cell surface but rather to a transcription blockade (Ulcer, Bartsch, Scheurich & Pfizenmaier, 1985).

Normal breast epithelial cells and their malignant counterparts were found to express very low levels of class II molecules (Bernard, Maurizius, Chassagne, Chollet & Plagne, 1985), whereas lactating breast cells which are at an ultimate differentiation stage strongly express these determinants (Newman, Ormorob & Greaves, 1980).

Because class I and class II molecules, and their relative expression, play a crucial role for the immune recognition of tumors and their metastases (Goodenow, Vogel & Linsk, 1985), and because DR expression may be important during breast cancer differentiation, we studied here the quantitative expression and kinetics of class I and class II molecules under rγIFN treatment using two human breast carcinoma cell lines. The biochemical analysis of DR molecules showed a normal protein expression but suggested a defective pattern of sialialation for one of the cell lines.

The heterogeneity and kinetics of the response in different cell lines may be of importance for the study of the host immune response.

EXPERIMENTAL PROCECURES

Cell lines

Two well characterized human breast carcinoma cell lines were studied. T47-D cell line was derived from a metastatic pleural effusion (Keidar, Chen, Karby, Weiss, Delarea, Radu, Chaitcik & Brenner, (1979) and was kindly provided by Dr J. Minna of the National Cancer Institute, Bethesda MD, U.S.A. H466-B cell line was derived from a malignant ascitic effusion from a patient with a very aggressive metastatic breast carcinoma and has been established and characterized in our laboratory, T47-D possesses estrogen and progesterone receptors, while H466-B does not. Both cell lines were free of mycoplasma contamination. T47-D is routinely maintained in 75 cm^2 Falcon plastic flasks at 37°C, in a 5% CO_2, 95% air humidified incubator, in DMEM (Gibco, France) supplemented with 10% heat inactivated fetal calf serum (FCS). H466-B cell line was cultivated in a medium (DMEM/F 12, 1:1) supplemented with hormones and growth factors (Calvo, Brower & Carney, 1984), containing insulin (3 μg/ml), transferrin (25 μg/ml), estradiol (10 nM), triiodothyronine (10 nM), dexamethasone prostaglandin F2 (100 ng/ml) (all from Sigma) and 5% fetal calf serum (FCS). Both cell lines were passaged weekly by trypsinization.

Reagents

Purified human recombinant gamma interferon with spec. act. of 10^7 U/mg protein was provided by Biogen laboratories.

Cell growth experiments

Growth experiments of the two cell lines were performed in triplicate in 25 cm^2 Falcon plastic flasks and repeated three times. Cells in log phase growth were harvested with trypsin EDTA (Gibco) after two washes in Ca- Mg-free PBS. Trypsin was neutralized with serum and 3×10^5 cells in 5 ml medium were plated with or without various concentrations of rγIFN.

B_2 microglobulin release

B_2 microglobuline (B_2M) release into the medium was tested using a radioimmunoassay kit (Commissariat à l'Energie Atomique, Saclay, France). Cell free supernatants at different days of culture were harvested, frozen, and stored at − 80°C until tested.

Immunofluorescence analysis of class I and class II antigen expression

Class I antigenic expression was determined using monoclonal antibodies recognizing B_2M (M − 18 antibody) (Fellous, Bono, Hyafil & Gresser, 1981) and a public determinant of HLA-A, B, C heavy chain (W6-32 antibody) (Barnstable, Bodmer, Brown, Galfre, Milstein, Williams & Ziegler, 1978)

Class II molecules were detected using a monoclonal antibody recognizing monomorphic DR antigens, D1 − 12 (Carrel, Tosi, Gross, Tanigaki, Carmagnola & Accola, 1981). Ascitis of the corresponding hybridoma was used as a source of antibody.

Binding was determined by indirect immunofluorescence using, as a second antibody, a FITC conjugated FAB'2 goat anti-mouse immunoglobulin antibody (Institut Pasteur, Paris, France). Nonspecific cell staining was controlled using a first monoclonal antibody of the same class, raised against Rotavirus. We used as a positive control, cells from a human B lymphoblastoid cell line which gave a strong 100% positive staining with M-18, W6-32 and D1-12 monoclonal antibodies. Briefly, 10^6 cells were labelled by a 30 min incubation at 4°C with 100 μl of the first antibody at 1/50 dilution in PBS-BSA 1%, sodium azide 0.02%. After three washes in the same buffer, cells were incubated for 30 min with the fluorescent antibody (100 μl, final dilution 1/50) washed three times and analyzed using an inverted Leitz fluorscence microscope. At least 200 cells were counted. For some experiments the analysis was controlled with flow cytometry (50 H Cytometer from Ortho instruments). DR positive cells were separated from the parental cell line H466-B by flow cytometry, amplified in culture and immunoprecipitated (see below).

Radio-immunoassay for semi-quantitative analysis of class I and class II antigen expression on live cells

A semi-quantitative assay was used to determine the heavy chain class I and class II molecules expressed on live cells. 10^6 cells were incubated at 4°C with various concentrations of W6-32 and D1-12 antibodies for 1 h. After three washes in PBS-BSA-Azide, 100 μl of ^{125}I iodinated FAB'2 fragment of goat anti-mouse immunoglobulin (New England Nuclear, U.S.A.) was added for 1 h at 4°C at a final dilution of 1/50. After three washes in the above buffer, bound radioactivity on cells was counted in a gamma counter. Unspecific binding of the anti-rotavirus antibody was subtracted and saturation

curves were plotted. All assays were performed at least three times in triplicate.

Immunoprecipitation of class II molecules and two dimensional electrophoresis (2D-PAGE)

3×10^7 control cells and 3×10^7 cells treated for 48 h with 1000 U/ml recombinant interferon were incubated for 4 h at 37°C in two ml of methionine free RPMI 1640 supplemented with 5% dialyzed FCS and 300 μCi of 35S-methionine (Amersham, U.K.); washed radiolabelled cells were extracted with 400 μl of 0.5% nonidet P-40 (NP 40) for 30 min at 4°C and extracts were then centrifuged at 11,000 g for 5 min. 400 μl of the NP-40 extracts were precleared with 400 μl of *Straphylococcus aureus cowan* I strain protein A (STAPH-A) for 30 min at 4°C. Specific radiolabelled cell proteins were then immunoprecipitated by the addition of 10 μl of D1-12 monoclonal antibody to 400 μl of STAPH-A precleared extracts for 120 mn. Antigen antibody complexes were eluted with 30 μl of electrofocusing sample buffer. Supernatant was kept frozen at −80°C until use. Samples were prepared according to the technique described by Laemmli (1970) and adapted by O'Farrel (O'Farrel, 1975; O'Farrel, Goodman & O'Farrel, 1977). In the first dimension, the proteins were separated according to their charges using a non equilibrium pH gradient electrophoresis (NEPHGE). The second dimension was run in 10% acrylamide slab gel (Charron & McDevitt, 1980).

RESULTS

Cell growth experiments

Because interferon has antiproliferative activity we tested the effects of rγIFN on the growth of two human breast carcinoma cell lines (Fig. 1). No growth effects of rγIFN were seen with H466-B cell line. There was an increase in the doubling time of the T47-D cell line in the presence of 5000 U/ml (36 h vs 30 h for control) which was not related to cell death increase as shown by trypan blue exclusion. Lower doses did not significantly modify the growth of this cell line.

HLA class I expression

Because HLA class I antigen is composed of a light chain (B_2M) and a heavy chain the effects of interferon were studied on both molecules.

B_2M release of the two cell lines. The release of B_2M was measured in the absence or the presence of

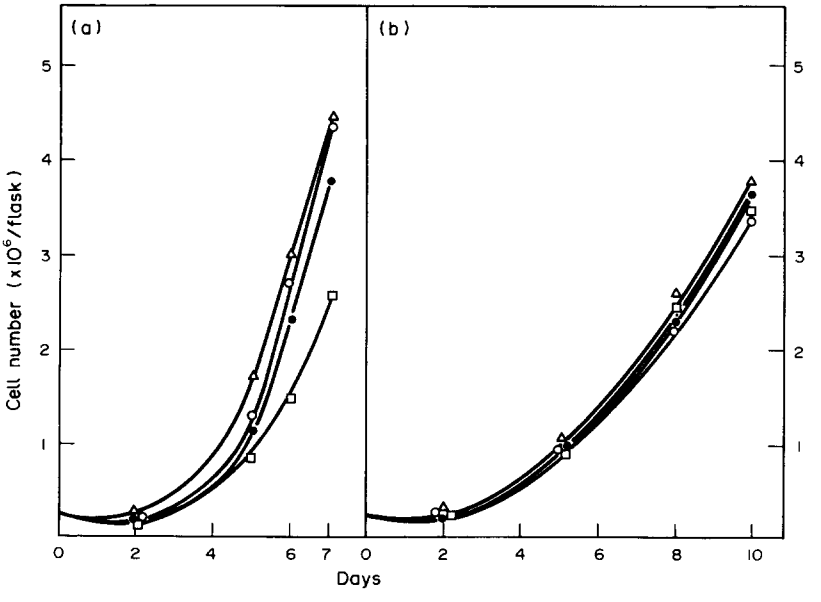

Fig. 1. Growth curves of (a): T47-D cell line and (b) H466-B cell line. On day 0, 3×10^5 cells in 5 ml of medium were plated.

Δ Control, ○ 100 U/ml, ● 1000 U/ML, □ 5000 U/ml rγIFN. Points are average values for triplicate cultures. Standard deviations are usually less than 10%.

rγIFN (Fig. 2). H466-B cell line spontaneously released B_2M and this was not modified by interferon. Untreated T47-D cells did not release detectable amounts of B_2M, but in the presence of rγIFN, the cells secreted B_2M, in a dose dependent manner; a more than three fold increase was observed with 5000 U/ml when compared to 100 U/ml for a constant cell number after 6 days in culture.

B_2M and heavy chain class I expression. 100% of T47-D and of H466-B cells were positively stained with M-18 and W6-32 monoclonal antibodies. After treatment with rγIFN, no difference in staining was observed with H-466B but T47-D showed a stronger fluorescence intensity. Radioimmunoassay on live cells confirmed these data (Fig. 3): a two fold increased binding of W6-32 monoclonal antibody on T47-D with 1000 U/ml rγIFN compared to control cells, while the H466-B binding was not modified with interferon.

HLA class II expression

Using fluorescence microscopy T47-D cells did not show any expression of class II antigens on their surface, while H466-B, on different passages during 1 yr, bound D1-12 on 8 ± 4% cells. These results were confirmed by flow cytometry. Under treatment with various doses of rγIFN, no significant modification of class II expression was seen with H466-B cells. On the other hand, T47-D cells, expressed strong fluorescence staining on 100% cells, as soon as 24 h after treatment with rγIFN. The use of radio-immunoassay on live cells showed that this expression was dependent on the rγIFN concentration (Fig. 4). The D1-12 antibody binding was increased by 30% with 1000 U/ml when compared to treatment with 100 U/ml. With 5000 U/ml, there was an eight fold increased binding compared to control cells. Kinetic studies of DR expression showed that the maximal increase with 5000 U/ml rγIFN was obtained at days 2–3, followed by a progressive decrease to the basal level, at day 12. Daily addition of 100 U/ml rγIFN, progressively increased DR expression, which reached the level obtained with 5000 U/ml once, after 5 days (not shown).

The D1-12 monoclonal antibody was used to immunoprecipitate class II molecules of untreated and rγIFN treated T47-D and H466-B cell lines and 2D-PAGE was performed (Fig. 5). It failed to show any spot in the MW range corresponding to class II molecules with H466-B and untreated T-47D. In contrast, after 48 h treatment with 1000 U/ml rγIFN, two dimensional electrophoresis in T47-D revealed the profile of human DR heterodimer molecules associating a 32–34 kD acidic alpha chain, a more basic 27–29 kD beta chain and a 31 kD molecule corresponding to the invariant chain (Ii). While

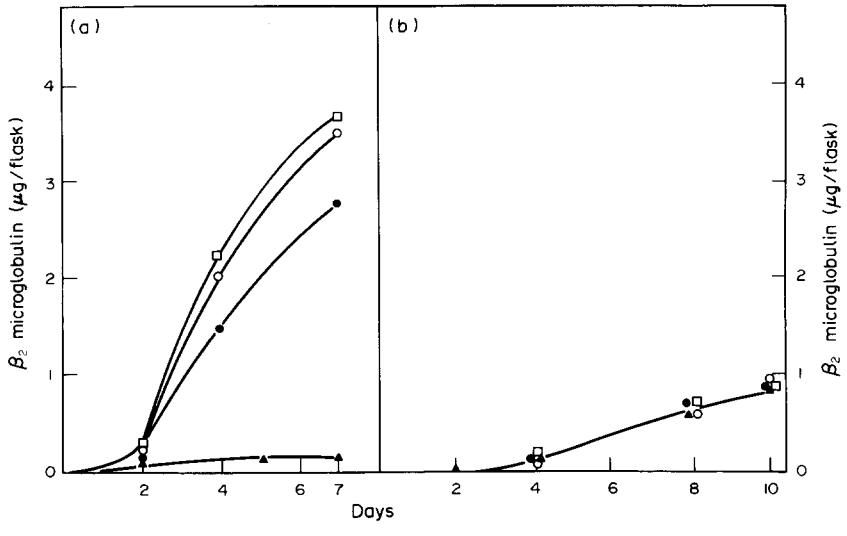

Fig. 2. B_2 microglobulin release in the culture medium of A: T47-D cell line and B: H466-B cell line. On day 0, 3×10^5 cells in 5 ml medium were plated. Medium was removed at the indicated days and assayed (see Experimental Procedures). ▲ Control, ● 100 U/ml, ○ 1000 U/ml, □ 5000 U/ml rγIFN.

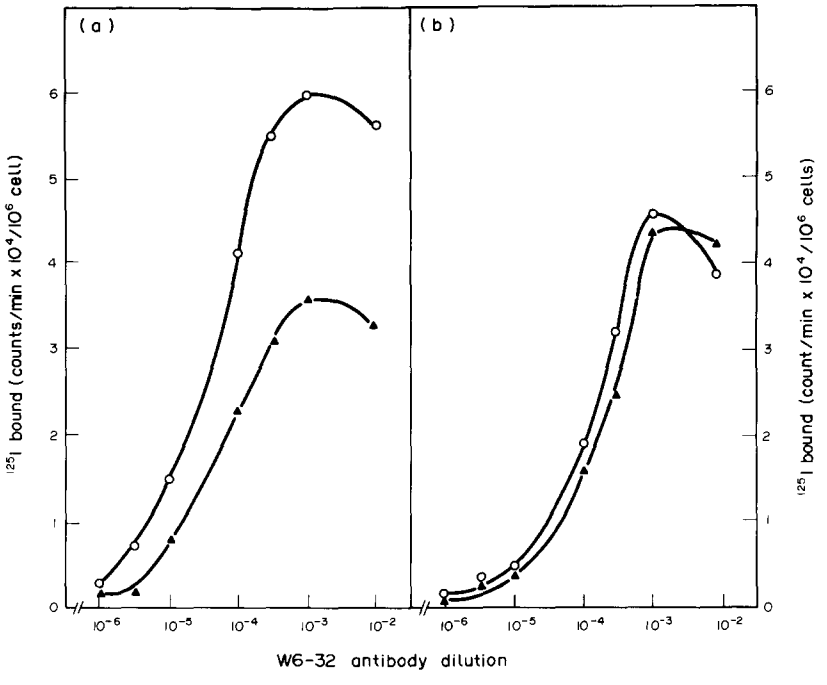

Fig. 3. Binding of monoclonal antibody (W6-32) to class I heavy chain antigen of (a) T47-D cell line and (b) H466-B cell line, as a function of antibody dilution. Points represent the average of triplicate determinations in the 125 I goat antimouse FAB'2 antibody binding assay. Cells were cultured for 48 h before tested. Background counts of non specific binding of anti rotavirus monoclonal antibody (Ig G) were subtracted.
Δ Control, ○ 1000 U/ml.

invariant chain was synthetized at a normal rate, displaying a set of spots comparable to those immunoprecipitated in lymphoblastoid cell lines, single alpha and beta spots were observed. This could be in favor of an incomplete sialialation of alpha and beta chains synthetized under rγIFN treatment. Conversely the DR positive enriched fraction of untreated H466-B cells showed a normal pattern of Ii, alpha and beta chains.

DISCUSSION

The modulation of class I and class II antigens in tumor cells may play an important role in either immune recognition, and/or in tumor cell differentiation. This study aimed at examining the effects of rγIFN on the expression of class I and class II molecules on two human breast cancer cell lines. The results are summarized in Table 1. Interferon did not modify the secretion of B_2M or the expression of class I and class II molecules on the surface of the H466-B cell line. On the contrary, there was a considerable stimulation of B_2M secretion and of expression of MHC molecules on the T47-D cell line, and these effects were dose dependent.

It has been shown that class I antigens play a critical role in the presentation and recognition of tumor cells (Zinkernagel & Doherty, 1979). Moreover, the expression of transfected class I genes is able to reverse the tumorigenicity of transformed cells which do not display surface class I antigens (Wallich, Bulbuc, Hammerling, Katzav, Segal & Feldman, 1985). The modulation of class I expression by interferons may thus play a major role in the enhancement of the immune response Hayashi, Tanaka, Jay, Khoury & Jay, 1985). The enhanced expression of B_2M and class I heavy chain in T47-D cells was an early event, affecting the whole cell population within 24 h after rγIFN addition. The strong effect on B_2M release (30 fold increase), while the expression of class I heavy chain was enhanced to only twice the control, suggests that the regulation of the synthesis of the two proteins is not correlated (Fellous *et al.*, 1981). B_2M can be the component of other membrane antigens as was shown in mice (Yokoyama, Stockert, Old & Nathenson, 1982).

Normal and malignant mammary glands do not usually express class II antigens, or only in very low amounts (Bernard *et al.*, 1985, Gasti, Marth, Leiter, Gattringer, Mayer, Daxenbichler, Flener & Huber, 1985, Natali, Giacomini, Bigotti, Imai, Nicotra, Ng & Ferrone, 1983, Newman *et al.*, 1980). During lactation these antigens are expressed in acini and found in milk fat globule membranes (Newman *et al* 1980). In animals, Ia antigenic expression has been shown to be induced by pregnancy, lactation and the exogenous administration of lactotropic hormones in virgin mice (Klaregskog, Forsum & Peterson, 1980). Therefore, class II antigenic expression might be correlated to differentiation state and hormonally regulated. Of the two cell lines studied here, only H466-B was shown to spontaneously express Class II molecules on a cell subpopulation. Heterogeneity of DR expression in fresh tumors and in cell lines has been already reported (Natali *et al.*, 1983; Daar & Fabre, 1983). By cell sorting we have enriched the class II positive cell fraction of H466-B (H466-B/DR+). When injected into nude mice, H466-B parental cell line and H466-B/DR+ cells, yielded tumors with different pathological aspects. The former gave rise to an undifferentiated adenocarcinoma while the latter formed a tumor of a more differentiated aspect with lobular organisation and gland-like cavities. Cytochemical staining revealed that the more differentiated aspect was associated with the presence of acid and neutral muccopolysaccharids (manuscript submitted for publication). Induction of class II antigens with interferon may thus have implications in the induction of differentiation. We are currently testing the effect of rγIFN treatment on the state of differentiation of tumors obtained in nude mice after injection of T47-D cells.

It has been shown that class II antigens allow the antigenic recognition of tumor cells and can modify the recognition of tumor associated antigens by the immune system (Goodenow, Vogel & Linsk, 1985). Interferon treated cancer cells lines and fresh tumor samples also showed an enhancement of proliferative cytolytic activity of T cells in allogenic and autologous mixed lymphocytes – tumour reaction (Pfizenmaier *et al.*, 1985). Working with mutant cell lines, others have shown that deletion of Ia alloantigens is associated with a loss in immunogenicity of

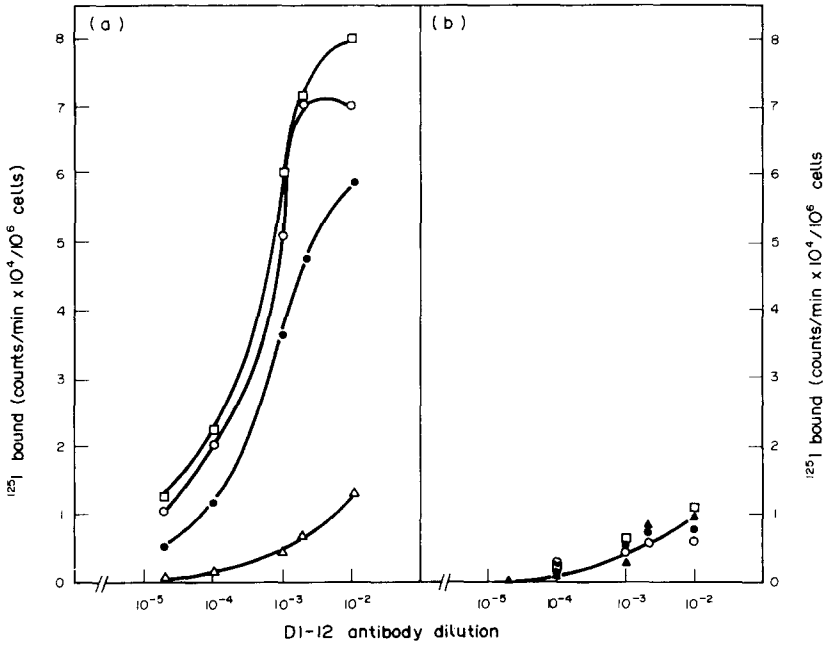

Fig. 4. Binding of monoclonal antibody (D1-12) to class II antigens of A: T47-D cell line, and B: H466-B cell line as a function of antibody dilution. Points represent the average of triplicate determinations in the ^{125}I goat antimouse FAB'2 antibody binding assay. Cells were cultured for 48 h before testing. Background counts of non specific binding of anti-rotavirus monoclonal antibody (Ig G) were subtracted.
△ Control, ● 100 U/ml, ○ 1000 U/ml, □ 5000 U/ml rγIFN.

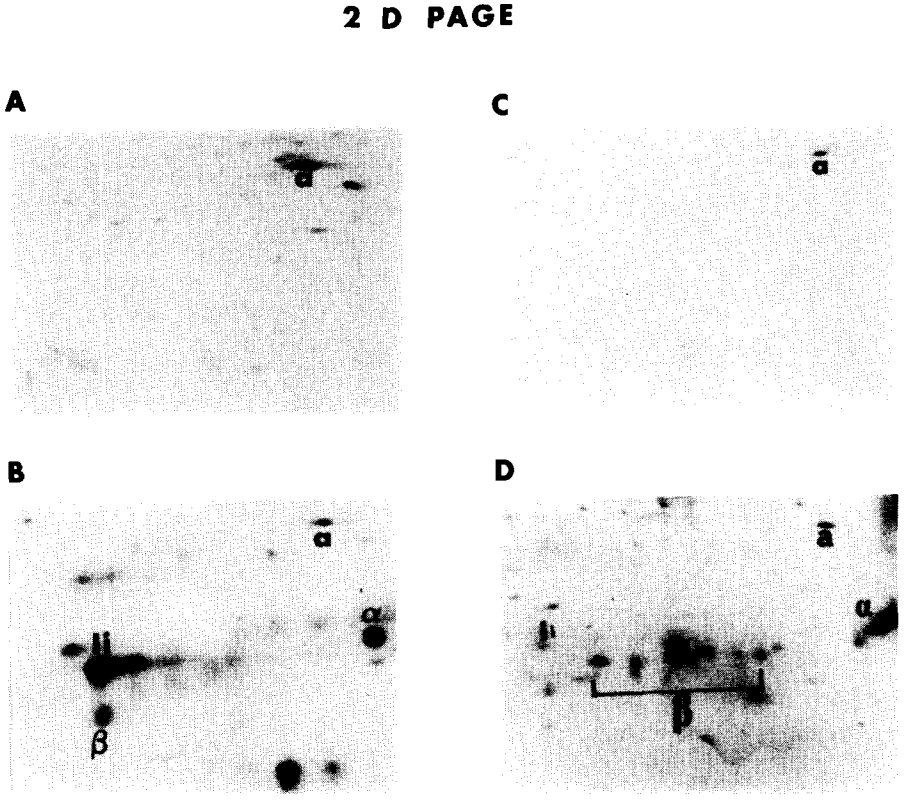

Fig. 5. Two-dimensional gel electrophoresis pattern of class II antigens from T47-D and H466-B cell lines immunoprecipitated with D1-12 monoclonal antibody. (A) Untreated T47-D cells. (B) 1000 U/ml rγIFN T47-D treated cells. (C) Untreated parental H466-B cells. (D) Untreated H466-B/DR enriched cells. α, designates actin, α designates the 34 kD acidic alpha chain on the right (acidic area); Ii designates the 31 kD invariant chain; the beta chain area of the gel displays only one spot for rγIFN T47-D treated cells.

Table 1. Effects of rγIFN on class I and class II antigen expression.

	Cell lines			
	T47-D rγIFN treatment (48 h; 1000 U/ml)		H466-B rγIFN treatment (48 h; 1000 U/ml)	
	−	+	−	+
B_2M expression*		+ + +	+	+
B_2M release†	−	+ + +	+	+
Class I heavy chain expression*		+ + +	+	+
Class II expression*	−	+ + +	8+4%	8+4%

*Fluorescence intensity as revealed by light microscopy of indirect immunofluorescence with monoclonal antibodies to B_2M (M18), class I heavy chain (W6-32), class II (D1-12).

†B_2M release in the medium as measured by radioimmunoassay.

+ Weak fluorescence.

+ + + Strong fluorescence. >95% cells were stained (using flow cytometry) unless otherwise stated.

tumor associated antigens (Forni, Shevach & Green, 1976). No class II expression was shown with untreated T47-D cells in our experiments. As for B_2M and class I heavy chain, class II molecules were strongly expressed as soon as 24 h after rγIFN addition. This expression progressively decreased when the culture medium was interferon depleted, and returned to the basal level after 12 days (not shown) according to published results (Gasti *et al.*, 1985). Two dimensional electrophoresis analysis of DR molcules synthetized under rγIFN treatment, revealed incomplete alpha and beta chain biochemical expression. It is known that for a given haplotype, beta chain is resolved in three or four spots on 2D-PAGE. The pattern of beta chain observed, suggest a defective biosynthesis. We still do not know if this incomplete expression affects the immune recognition of the cell. This incomplete class II molecule biosynthesis has also been shown in a Burkitt subline induced to differentiate with sodium butyrate (Spiro, Sairenji & Humphreys, 1984).

In conclusion, our study has shown that human breast cancer cell lines are heterogenous in their expression of class I and class II antigens and in their response to rγIFN. These results and ongoing studies indicating that expression of class II molecules is associated with differentiation characteristics may be useful for the design of clinical trials with rγIFN.

Acknowledgements — We are grateful to Dr C. J. Larsen and Dr. V. Lepage for helpful discussions, Drs J. Colombani, D. Charron and A. Rosetto who provided us with monoclonal antibodies, and Mr R. Miglierina (Service commun du trieur de cellules, Hôpital St Louis) who performed the experiments on flow cytometry.

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