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HLA expression in hepatocellular carcinoma cell lines

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SUMMARY

The present study undertook to investigate the biological significance of human leucocyte antigen expression in hepatocellular carcinoma and to elucidate the role of potential modulating agents on human leucocyte antigen expression. These studies used several hepatic tumour-derived cell lines as *in vitro* model systems. The cell lines included PLC/PRF/5 (Alexander cell line), Hep3B, HepG2, TONG PHC, HA22T/VGH, HA59T/VGH and Mahlavu. The cell lines K562 and Raji were used as negative and positive controls, respectively. K562, a B lymphoid-derived cell line, was shown to express negligible amounts of human leucocyte antigens, while Raji, an erythromyeloid-derived cell line, expressed both class I and class II human leucocyte antigens as well as their respective invariant chains, β_2-microglobulin and Ii. Using an ELISA, experiments performed on these cell lines confirmed the natural expression of class I and class II antigens by the HA22T/VGH and HA59T/VGH cell lines, whereas PLC/PRF/5 displayed class II surface antigens only. The effects of modulating agents such as interferon-gamma sodium butyrate and clofazimine on human leucocyte antigen expression were investigated using the HA22T/VGH, HA59T/VGH and TONG PHC cell lines. These agents increased class I and class II human leucocyte antigen expression on HA22T/VGH and TONG PHC cells, but had no effect on the HA59T/VGH cell line. The results suggest a potential use for these agents as modulators of human leucocyte antigen expression by human heptocellular cell lines.

Keywords HCC cell lines HLA expression

INTRODUCTION

Few or no class I and class II human leucocyte antigens (HLA) appear to be expressed by normal hepatocytes [1]. It is postulated that the variable findings that have appeared in the literature in recent years may represent unrecognized immunological agents promoting the expression of HLA products at the hepatocyte membrane [2]. HLA antigen expression in many liver diseases has been well documented. These include hepatitis B virus infection [3–5] and hepatocellular carcinoma (HCC) [2,5–7]. Paterson *et al.* [2] have reported a homogeneous acquisition or enchancement of MHC class I with concomitant expression of the invariant chain, β_2-microglobulin (β_2-M), in the majority of cases.

Several human HCC or hepatoblastoma (HB) cell lines are available. These may be useful tools for the study of MHC gene expression and regulation in hepatocytes as well as for the investigation of MHC antigens with transformation of human hepatocytes [5,7,8]. Most of these cell lines have been at least partially characterized for the presence or absence of MHC antigens, although results have sometimes been at odds [9,10]. Since HCC cell lines exhibit various hepatospecific phenotypes [11] they can be studied for their expression and regulation of hepatocyte markers *in vitro*.

Agents such as interferon-gamma (IFN-γ) and sodium butyrate had previously been shown to enhance the expression of HLA class I and II expression by HCC cell lines [9,12]. Furthermore, it has been well documented that clofazimine, an effective anti-leprosy agent [13,14], stimulates phagocytic and microbicidal activities [15,16] and potentiates macrophage intracellular killing mechanisms [17]. A further aspect of this study therefore was to examine the effects of these immune response modifiers on HLA class I and class II antigens on the various HCC cell lines.

Quantification of cell surface antigens could be useful to evaluate cell activation [18]. Generally the techniques employed for the detection of such antigens are direct immunofluoresence assays, flow cytometric techniques or radioimmunoassays. Even though these techniques are easy to perform and may provide rapid results, their usefulness is limited to large numbers of cells in suspension. Since some of the cell lines used in the current study only grow as monolayers, the assessment of antigens being expressed on their surfaces using

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the above techniques could be compromised by the various procedures used to suspend cells. For these reasons, and because fixation has been shown not to affect the antigens on an HCC cell line PLC/PRF/5 [19], the present study evaluated the expression of HLA class I and class II antigens under normal and stimulated conditions using a cell-ELISA. With regard to the latter, our studies assessed the possible use of chemical and physiological modulators in enhancing the expression of HLA on various cell lines.

MATERIALS AND METHODS

The cell lines assessed were either microscopically epithelial- or fibroblast-like in appearance. Those in the first category were the Alexander cell line PLC/PRF/5, HepG2, Hep3B and TONG PHC. The fibroblast-like cell lines used in this study were the HA22T/VGH, HA59T/VGH and Mahlavu lines. The cell lines HA59T/VGH and Mahlavu were obtained from Highveld Biologicals (Johannesburg, South Africa) and HA22T/VGH as a gift from Dr S. Aspinall, (Medical University of South Africa). In addition, the Raji cell line established from the tumour tissue of a Burkitts' lymphoma [20], which expresses both class I and class II antigens [21], and the K562 erythromyeloid cell line [21], which expresses neither class of antigen naturally, were used as positive and negative controls, respectively (Highveld Biologicals). On examination, all cell lines were found to be free of mycoplasma, as assessed by the Hoescht DAPI fluorescent stain (Highveld Biologicals).

The cultures of each cell line were grown in disposable 25 and 75-cm^3 culture flasks (Cel-Cult, Sterilin, Hounslow, UK) in a humidified 5% CO_2, 95% air incubator at 37°C. Cells from confluent cultures were disaggregated into a single-cell suspension by incubation with 0·25% trypsin and 0·01% EDTA at 37°C for 30 min. Trypsinization was terminated by the addition of fresh medium containing 10% fetal calf serum (FCS) at 37°C, after which the cells were harvested by centrifugation for 5 min at 200 *g* and resuspended in fresh medium. Cells grown in monolayers had a viability of 85–90%, while those grown in suspension demonstrated cell viability always greater than 95%.

Antibodies

The primary antibodies used in this study were directed against (i) the monomorphic determinants of HLA-A, -B, -C (W6/32; Dakopatts, Glostrup, Denmark); (ii) the heavy non-polymorphic chain of HLA-DR (Tal IB5; a gift from J. Bodmer, ICRF, UK); (iii) the HLA class I-associated invariant chain β2m (anti-β_2-M; Dakopatts); and (iv) the HLA-D region-associated invariant chain, Ii (Vic Y1; An-Der-Grub Bioresearch, Kaumberg, Austria). The secondary antibody used in these assays was a peroxidase-conjugated goat anti-mouse antibody (Cappel Labs, Cochranville, PA).

IFN-γ (Amersham International, Aylesbury, UK) and the chemicals sodium butyrate (Merck Chemicals, Munich, Germany) and clofazimine were prepared as stock solutions, maintained in RPMI 1640 (Highveld Biologicals), filter sterilized through 0·22-μm filters (Millipore, Bedford, MA) and the resulting solutions stored at 4°C until used. Clofazimine (a gift from Professor R. Anderson, University of Pretoria, South Africa), sodium butyrate and recombinant IFN-γ were used at final concentrations of 0·1, 1, 2, 5 μg/ml; 1, 2 and 5 mM and 50, 100, 200 U/ml, respectively.

Determination of MHC antigen expression on HCC cell lines by ELISA

The ELISA was performed in flat-bottomed 96-well culture plates (Cel-Cult Sterilin) using a modification of the technique described by Nibbering *et al.* [18]. All procedures were performed at room temperature unless otherwise stated. Ten thousand cells of the various HCC cell lines in RPMI containing 10% FCS (cRPMI) were added to each well and the plates incubated at 37°C in 95% air, 5% CO_2. After 24 h the cells were found to have adhered to the bottom of the well and had reached 85% confluency. Thereafter the cells were fixed for 30 min at room temperature with 100 μl of 0·5% paraformaldehyde. After three washings with PBS-Tween, unbound sites on the surface of each well were blocked with 0·5% bovine serum albumin (BSA) in carbonate buffer, at pH 9·6, and the plates were incubated at room temperature for 60 min. The plates were washed four times with PBS-Tween and 100 μl of an appropriately diluted primary antibody in PBS-Tween then added to each well. After incubation at room temperature for a further 2 h, followed by four washings with PBS-Tween, the cells were incubated with 100 μl of the enzyme-labelled antibody appropriately diluted with PBS-Tween. Thereafter, cells were incubated in the dark with 100 μl of a peroxidase substrate, *o*-phenylenediamine (OPD), in the presence of a 0·03% H_2O_2 substrate buffer. The reaction was terminated after 15 min with 50 μl of 2·5 M H_2SO_4. The optical density (OD) of each well was read at a wavelength of 492 nm on an ELISA Multiskan Titertek Spectrophotometer (Flow Labs, Irvine, CA).

The ELISA was standardized in terms of concentration of the primary and secondary (enzyme-labelled conjugate) antibodies. To this end checkerboard ELISAs were performed in order to establish the optimal dilution of the two antibodies required for each cell line. In these experiments the primary antibodies, W6/32 (anti-class I), anti-β_2-M and TAL IB5 (anti-class II) were used at dilutions of 1 : 10, 1 : 50, 1 : 100 and 1 : 200 in PBS-Tween, while VicY1 (anti-Ii) was used at 1 : 500, 1 : 1000, 1 : 1500 and 1 : 2000.

In addition, enzyme-labelled conjugate at various dilutions was added to the wells, such that each of the dilutions of the MoAb was incubated with each dilution of the secondary antibody.

Since Raji and K562 cells were maintained as suspensions, these cells were plated at a concentration of 1×10^4 cells/well in 100 μl of cRPMI. The microtitre plates were immediately centrifuged at 200 *g* in a modified plate centrifuge for 5 min. The supernatant was gently aspirated, the cells fixed as described and used in ELISA as for other cell lines.

The effects of IFN-γ, clofazimine and sodium butyrate on HCC cell line expression of MHC antigens

For these experiments, three of the best growing cell lines, HA22T/VGH, TONG PHC and HA59T/VGH, were used. These cell lines also represented the varying degrees of HLA class I (Fig. 1), and class II (Fig. 2) antigens that were expressed naturally. Previously determined optimized and non-toxic doses of the lymphokine rIFN-γ (100 μg/ml) and the chemicals clofazimine (1 μg/ml) and sodium butyrate (2 mM) were added to wells and plates incubated at 37°C in an humidified (95% air, 5% CO_2) chamber. In order to determine the optimal time required for maximal expression of HLA antigens by the

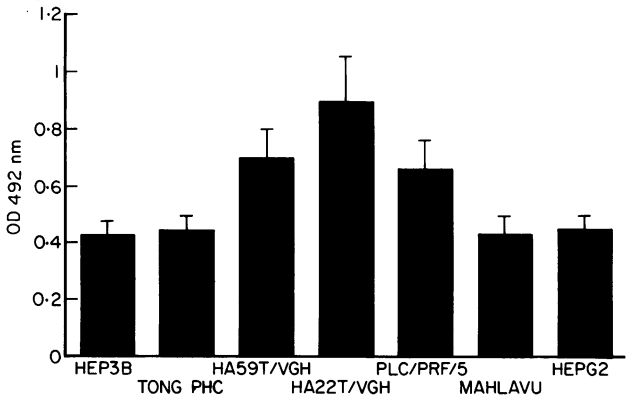

Fig. 1. Natural expression of HLA class I antigens by various hepatocellular carcinoma (HCC) cell lines. HLA expression was determined by a cell-ELISA where raised OD values were suggestive of increased HLA expression (mean ± s.d. of five experiments).

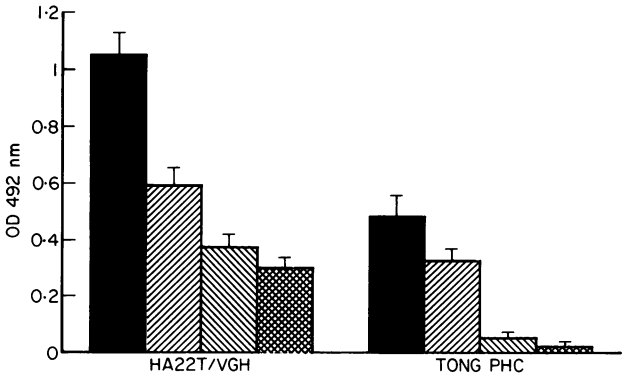

Fig. 3. Expression of invariant chains associated with HLA class I and II antigens. The cell lines were chosen specifically for their ability to express naturally HLA class I (■; HA22T/VGH and TONG PHC) and class II (▨; HA22T/VGH only). See text for details. (Mean ± s.d. of five experiments.) ▨, β_2-microglobulin; ▩; Ii.

various cell lines, plates were removed from the incubator at 4, 18, 24, 48, 72 and 96 h and the cells fixed with a final concentration of 0·25% paraformaldehyde. The plates were incubated at room temperature for 30 min and thereafter at 4°C until used (maximal time was 96 h). All ELISAs were performed simultaneously.

RESULTS

Expression of HLA class I antigens by HCC cell lines

Figure 1 (mean ± s.d. of five experiments) demonstrates that all of the seven HCC cell lines assessed express HLA class I antigens naturally. The amount of class I antigen expressed as recorded by binding of enzyme-labelled secondary antibodies varied between each cell line. The qualitative detection of class I antigens was optimal when the primary and secondary antibodies were used at dilutions of 1:50 and 1:500, respectively.

Using similar techniques with anti-class II antisera, natural expression of class II antigens was restricted to some cell lines only (Fig. 2). HLA class II expression, reflected by raised OD levels, could be detected on the cells derived from PLC/PRF/5, HA59T/VGH and HA22T/VGH cell lines only. In comparison,

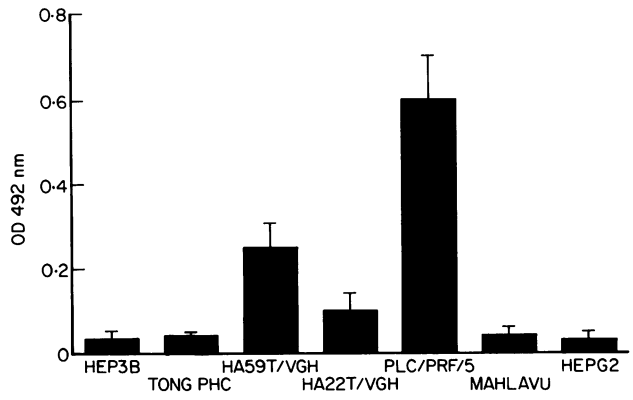

Fig. 2. Natural expression of HLA class II antigens by various hepatocellular carcinoma (HCC) cell lines (mean ± s.d. of five experiments).

very low or negligible amounts of class II antigens could be detected on cells from the cell lines TONG PHC, Hep3B, HepG2 and Mahlavu. When these latter cell lines were used in assays employing varying concentrations of primary and secondary antibody (higher and lower), no increase in the detection of class II antigens was observed (results not shown).

When cell lines expressed class I antigens the associated invariant chain, β_2-M, could also be detected. Similarly, when HLA class II antigens were expressed naturally, the corresponding invariant chain, Ii, associated with these antigens could also be detected. Figure 3 demonstrates the results of one such investigation using the cell lines HA22T/VGH and TONG PHC as examples (mean ± s.d. of five experiments). Both class I and II antigens are expressed by HA22T/VGH cells. Correspondingly, β_2-M and Ii could also be detected on their cell surfaces. However, even though TONG PHC cells expressed appreciable levels of class I and β_2-M, negligible amounts of class II or the associated Ii could be detected on these cells (Fig. 3).

Detection of HLA antigens on Raji and K562 cell lines

The erythromyeloid cell line K562 naturally expressed very low levels of class I and class II antigens (Fig. 4). Since the OD values in these assays were not greater than 0·04 (including standard deviations), any value below this was regarded as an absence of natural HLA expression. Values greater than this were taken to indicate appreciable natural expression of HLA antigens. These results therefore suggest that K562 cells do not express HLA antigens naturally. Figure 4 also demonstrates the natural expression of HLA class I and class II antigens as well as their associated invariant chains, β_2-M and Ii, respectively, on the Raji cell line. Expression of such antigens as detected by OD values suggests Raji cells express high amounts of these antigens naturally.

The effects of IFN-γ, sodium butyrate and clofazimine on the expression of HLA antigens by various cell lines

The results indicate that the expression of HLA class I and class II antigens on the cell lines HA22T/VGH and TONG PHC could be enhanced by the addition of clofazimine, sodium

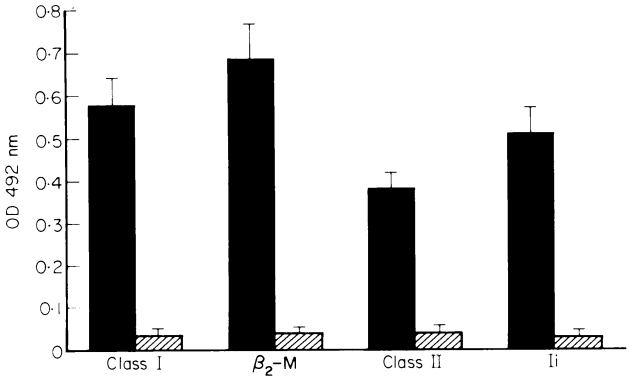

Fig. 4. Natural expression of HLA class I and class II antigens by Raji (■) and K562 (⬚) cell lines. Expression of class I and class II antigens could only be observed on cells from the Raji cell line as detected by the cell-ELISA. (Mean ± s.d. of five experiments.)

butyrate or IFN-γ for 24 h (Fig. 5). Clofazimine induced moderate increases of class I but significant increases ($P < 0{\cdot}01$) of class II expression by these two cell lines. IFN-γ and sodium butyrate significantly increased both class I ($P < 0{\cdot}005$) and class II ($P < 0{\cdot}01$) expression. Increased HLA expression could be observed as early as 18 h and remained elevated for up to 72 h (results not shown). The cell line HA59T/VGH, which naturally expressed both class I and II antigens, appeared to be refractory to the use of any of these agents (Fig. 5). The levels of class I and class II antigens that could be detected remained unaltered, even with increasing concentrations or longer incubation periods (results not shown).

DISCUSSION

The present study demonstrated that all HCC cell lines investigated naturally express class I and β_2-M (Figs 1 and 3). Even though our results are qualitative, they are consistent with those reported previously [5,7,9,10]. Expression of class II antigens was seen on some, but not all, of the cell lines assessed (Fig. 2). This is in keeping with previous reports on the expression of HLA class II antigens [5,7,9]. The cell lines which did not naturally express class II antigens were HepG2, Hep3B and TONG PHC. In the current study, PLC/PRF/5 cells expressed relatively high levels of HLA class II antigens naturally compared with the other cell lines assessed (Fig. 2). Whilst other workers had detected negligible amounts of class II antigens on the surface of PLC/PRF/5 cells [7,9], others demonstrated increased class II expression [10]. It is possible that the low level of class II detected could be due to a reduced sensitivity of the immunofluorescent assay used by these investigators. In the present study, the ELISA appeared to be far more sensitive than the immunofluorescent assays, even though detection of HLA correlated between the two assays (results not shown). It has been documented that fixation of cells has no appreciable effect on the reactivity of cell surface antigens [19,22] to monoclonal and polyclonal antibodies used in ELISA when compared with flow cytometric analysis [19]. Notwithstanding, the discordance in these findings does not exclude the possibility of such antigens being expressed in greater concentrations following successive passaging. Further investigations using a cell line that expressed appreciable levels of both HLA class I and II antigens (HA22T/VGH) and another that expressed raised levels of HLA class I only (TONG PHC) demonstrated the appearance of the associated invariant chains (Fig. 3). Both β_2-M and Ii could be detected on the cells of HA22T/VGH, whereas only significant levels of β_2-M could be detected on the cells of TONG PHC. Interestingly, this cell line did not express raised levels of HLA class II antigens, and even lower amounts of the associated Ii could be detected on these cells (Fig. 3).

The validity of assigning positive and negative controls to the Raji and K562 cell lines respectively was confirmed by the ELISA (Fig. 4). HLA antigens and their associated invariant chains were detected only on the Raji cell line. This cell line has been consistently reported to express HLA class I and class II antigens naturally [21]. K562 has been typed as expressing no significant amounts of HLA antigens under normal control conditions [23]. Even though the results presented in Fig. 4 demonstrate the detection of antigens using optimized primary and secondary antibodies, the use of high amounts of these antibodies did not increase the OD values obtained for K562 (results not shown).

The effects of IFN-γ on HLA expression by HCC cell lines have been previously documented [12,19]. Such studies demonstrated increases in class I but not class II antigens [19], whilst

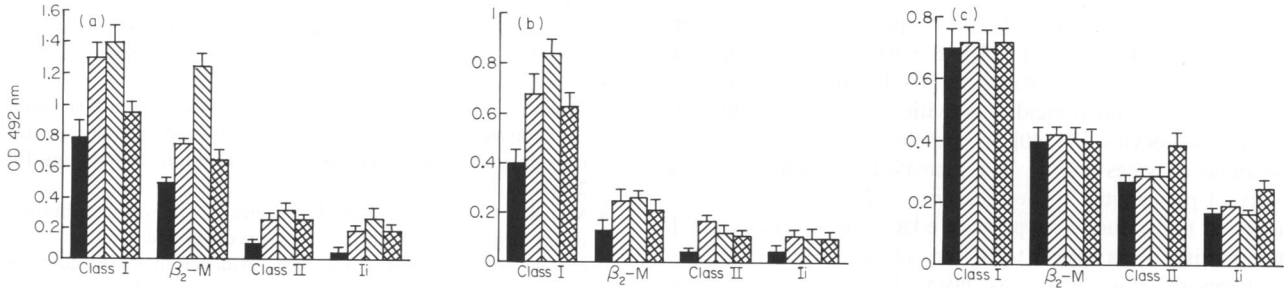

Fig. 5. Expression of HLA class I and II antigens by (a) HA22T/VGH, (b) TONG PHC and (c) HA59T/VGH cells incubated with IFN-γ, sodium butyrate and clofazimine. The figure suggests moderate to significant increases of HLA class I and II expression by HA22T/VGH and TONG PHC cells due to IFN-γ, sodium butyrate and clofazimine treatment. HLA expression by the cell line HA59T/VGH was not affected by any of these agents (see text for details). ■, Control; ⬚, IFN-γ; ⬚, sodium butyrate; ▨, clofazimine.

others demonstrated increases in class II antigen expression with this lymphokine [10,24]. The present study confirms that IFN-γ significantly enhances the expression of HLA class I and class II antigens and their associated invariant chains on HA22T/VGH and TONG PHC cell lines (Fig. 5). The expression of HLA class II antigens by HA22T/VGH cells has been shown to increase in response to IFN-γ [9]. The present study is in agreement with such an effect (Fig. 5). The expression of HLA class II antigens on the moderately defined TONG PHC cell line [25] was also increased in response to IFN-γ (Fig. 5).

Whilst it has been suggested that the level of class II antigen expression is inversely related to the degree of cellular differentiation [9], the sensitivity to IFN-γ may also depend on the number of respective receptors on cell surfaces [24]. This may account for the increased expression of class II antigens on some cell lines but not others (Fig. 5). Although class II antigens are not expressed by normal human hepatocytes, their expression in inflammatory liver diseases appears in the presence of IFN-γ [10,26]. Interestingly, intrahepatic lymphocytes increase their adhesiveness and release of IFN-γ during an inflammatory response [27,28]. This would lend support to the notion of possible immunotherapy with IFN-γ causing an increased expression of class II antigens by tumour cells, resulting in recognition by the host's immune system.

The physiological concentration of sodium butyrate, a non-toxic antiproliferative compound, is 2 mM [29], even though its effectiveness has been seen up to a concentration of 10 mM [30,31]. In the current study, sodium butyrate significantly enhanced the expression of class I and class II antigens and β_2-M of HA22T/VGH and TONG PHC cell lines, but appeared to have no effect on HA59T/VGH cells.

Sodium butyrate has been shown to include the differentiation of transformed cells in culture into more mature or 'normal' cells [32], to alter the growth rate of some cell lines [33,34], and to affect gene expression [30,35], including production of α-fetoprotein [33,36]. Such studies suggest its chemotherapeutic use in the treatment of solid tumours [32,34]. Since sodium butyrate affects different genes and different cell types in a tissue-specific manner, it may be capable of both inducing and/or repressing the expression of genes [30]. The results of the present study support this view, since an effect of sodium butyrate was seen in only two of the three cell lines assessed. It is possible that the negligible increase of class I antigens by sodium butyrate-treated HA59/VGH cells may be a reflection of the already high amount of class I antigens being expressed naturally by the cell line.

Clofazimine, also known as lamprene or B663, is an effective anti-leprosy agent [13,14,37] with antiinflammatory and immunosuppressive properties [38–40] that stimulates the phagocytic and microbicidal activities [15] of human and murine phagocytes [38–40] by potentiating the activity of lysosomal enzymes [16,41]. The increased intracellular killing ability of phagocytes in the presence of clofazimine has been suggested to be due to its priming effects on phagocytes [17], such that its effects appear to be very similar to that of IFN-γ. Furthermore, this agent has been shown to demonstrate antiproliferative effects on cancer cell lines, and behaves as an anti-neoplastic agent [42,43]. The present study therefore undertook to examine if clofazimine affected the expression of HLA antigens on HCC cell lines as seen with IFN-γ [10,19,24,44,45]. In addition, studies from this laboratory have suggested an increase in the expression of class II antigens by normal peripheral blood monocytes in culture (Wadee *et al.*, manuscript in preparation). The present study demonstrates that clofazimine increases the expression of class I and β_2-M on the cell lines HA22T/VGH and TONG PHC, and the expression of class II and its associated invariant chain, Ii, on HA22T/VGH, HA59T/VGH and TONG PHC cell lines. Such results suggest a role for clofazimine similar to that of sodium butyrate and/or IFN-γ. With regard to the latter, clofazimine has been shown to affect phagocyte function in a similar manner to that seen with IFN-γ [44,45].

Relatively few studies have investigated HLA antigen expression in large numbers of solid tumours. Paterson *et al.* [2] observed HLA class I expression in virtually all cases of human hepatocellular carcinoma and postulated that malignant transformation in the liver is characterized by expression of class I antigens that is at least enhanced, if not acquired *de novo*, in virtually all cases. Class II antigen expression was seen in nearly half of the cases, always associated with Ii display. It has been suggested that the ability to express class II antigens in response to appropriate stimuli is constitutive to all epithelial cells; but that it may be the failure to express antigen by a percentage of tumours which represents the aberrant state [46].

This study therefore provides a role for chemicals and lymphokines like clofazimine, sodium butyrate and IFN-γ in the expression of HLA antigens by HCC cell lines. Even though our investigations provide a qualitative assessment, our evidence confirms that all cell lines naturally express class I antigens. The expression of both class I and II antigens on two of the three cell lines assessed is increased in the presence of these substances. It is therefore suggested that these agents could play a role in modifying HLA expression by tumour cells, which could then result in an increased immune response, as has been suggested to occur on hepatocytes aberrantly expressing HLA class II antigens [26].

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