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Oxidative Stress Induces the Expression of the Major Histocompatibility Complex in Murine Tumor Cells

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The effect of *t*-butyl hydroperoxide (*t*-BOOH) on the induction of the Major Histocompatibility Complex (MHC) class I genes has been studied in two cell clones (B9 and G2) of the methylcholanthrene-induced murine fibrosarcoma GR9. These two clones were selected based on their different biological and biochemical behavior specially related to their tumor induction capability when injected into a BALB/c mouse. *t*-BOOH (0.125 mM) induced the expression of H-2 molecules in both cell clones. In B9 cell clone, in which MHC basal expression is very low or absent, *t*-BOOH significantly induced H-2K^d, H-2D^d and H-2L^d molecules. In G2 cell clone the expression of MHC class I genes was also enhanced by the xenobiotic, the effect being especially significant on the H-2L^d molecule which is not expressed under basal conditions. H-2 molecules expression was accompanied by the activation of the transactivator factor NFκB. These results suggest that oxidative stress may modulate the antigen expression of tumor cells and thus the immune response of the host organism.

Basal levels of oxidative parameters, such as anti-oxidant enzymes, malondialdehyde (MDA) and the DNA damaged base 8-hydroxy-2′-deoxyguanosine (8-OHdG), showed differences between the two fibrosarcoma cell clones.

Keywords: MHC class I antigens, fibrosarcoma, 8-OHdG, oxidative stress, NFκB

INTRODUCTION

During the past years, a great amount of information has been reported supporting the involvement of reactive oxygen species (ROS) in the regulation of metabolism and gene expression.[1,2] It has also been shown that moderate concentrations of intracellular ROS may influence the molecular machinery of gene expression and the translational modification of proteins by different mechanisms.[3] Important contributions in relation to the regulatory mechanisms involved in the adaptation of cells to oxidative stress,[4,5] together with the expression of different proto-oncogenes, heme oxygenase, apoptotic, growth arrest and DNA damage genes by intracellular redox modulation have

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been reported[6–14] and have been of great value for the understanding of the signal transduction pathways underlying the biological response to oxidative stress. In addition, two well-defined transcription factors, nuclear factor-κB (NFκB) and activator protein-1 (AP-1), have been implicated in the inducible expression of different genes triggered by changes of the redox state of the cells.[2] NFκB is a multisubunit transactivator factor that can rapidly activate the expression of genes involved in inflammatory, immune and acute phase responses[15] such as the MHC class I genes, whose expression in tumor cells under oxidative stimulus, has not been reported before. MHC class I genes represent a set of genes that synthesize products specializing in the processing and presentation of endogenous or exogenous antigens to the immune system. Due to the central role of MHC molecules in antigen presentation including tumor antigens,[16,17] any alteration in MHC class I gene expression may have profound implications in tumor development. Indeed, in a high percentage of human and murine tumors screened, complete locus[18,19] and/or allelic losses of HLA and H-2 expression have been described and recently reviewed.[20]

These facts prompted us to investigate the possible susceptibility of the histocompatibility antigens to an oxidative stress-induced modulation. We used as an experimental system, two cell clones from a well-characterized tumor model, GR9, which is a fibrosarcoma originated in a BALB/c mouse by subcutaneous (s.c.) injections of 0.2 mg methylcholanthrene.[21] Clonal heterogeneity of the chemical-induced GR9 fibrosarcoma has been described showing that different derived clones express quantitative variations of H-2 class I antigens. These variations range from H-2-negative clones to clones with strong expression while their local tumorigenicity or metastatic capacity has also been defined.[22]

We used two GR9 derived cell clones which were selected based on the different pattern of H-2 expression and on their tumorigenic potential in BALB/c mice. The GR9-B9 clone ($H\text{-}2^d$) negative and the GR9-G2 ($H\text{-}2^d$) positive have been used in this study and will be referred to in the text as B9 and G2 respectively. B9 and G2 clones have been characterized from an oxidative metabolism point of view and compared with normal BALB/c3T3 cells.

The reported results show first evidence of the induction of H-2 antigen expression ($H\text{-}2K^d$ $H\text{-}2D^d$ and $H\text{-}2L^d$) in two fibrosarcoma derived clones by *t*-BOOH. This effect is supported by the activation of NFκB by the xenobiotic with significant differences, in terms of the degree of antigens expression and time required, when compared with that observed with γ-interferon (γ-INF).

MATERIALS AND METHODS

Reagents

Superoxide dismutase (SOD), catalase (Cat.) and glutathione peroxidase (GPx) used as standards for the respective enzyme analysis were purchased from Boehringer (Mannheim, Germany). Cytochrome c, xanthine oxidase, EDTA, acrylamide/bisacrylamide, 2′-deoxyguanosine, SDS, bovine serum albumin (fraction V) and *t*-BOOH were from Sigma Chemical Co. (St. Louis, MO, USA). Reduced glutathione (GSH) was from Merck (Darmstadt, Germany). MoAbs and cell lines were obtained from the suppliers indicated below. Tissue culture medium and fetal bovine serum was from Gibco-BRL. Other reagents were of the highest purity available obtained from Chemical Companies.

Cell Lines, Culture Conditions and Analysis of Oxidative Stress Parameters

We used, in this work, two clones from the chemically-induced fibrosarcoma GR9, which was induced by s.c. injection of 0.2 mg of

methylcholanthrene into the flank of a BALB/c mouse. The tumor was adapted to tissue culture with no *in vivo* passage to avoid immuno selection, and further submitted to cell cloning.[21] GR9 clones were determined in terms of their H-2^d antigen expression (K^d, D^d and L^d molecules). Where indicated, non-tumorogenic BALB / c3T3 fibroblasts from the American Type Culture Collection (ATCC), Rockville, MD, were used in this study. Cells were grown in monolayers at 37 °C in a 5% CO_2 atmosphere and RPMI supplemented with 10% FCS, glutamine and antibiotics was used as culture medium.[21]

Cells grown until 90% confluency were washed twice with ice-cold PBS and collected by the addition of 5 ml 0.2% EDTA solution. Cell suspensions were transferred to plastic disposal tubes and sonicated for 2 min at 15 sec bursts, placed on an ice-containing tray and followed by 5 min centrifugation at 13,000 r.p.m. Appropriate aliquots were obtained for the respective analytic assays.

Analyses of Oxidative Stress-related Parameters

SOD activity was measured according to the method of McCord and Fridovich[23] based on the production of superoxide radicals during the conversion of xanthine to uric acid by xanthine oxidase and the inhibition of cytochrome c reduction. One unit of SOD activity was defined as the amount of SOD that produces 50% inhibition of cytochrome c reduction. Cat. and GPx activity were determined following the methods of Clairbone[24] and of Gunzler and Flohe[25] respectively. GSH content of cells was determined using previously described assay.[26] For the analysis of oxidized glutathione (GSSG) samples were treated with *N*-ethylmaleimide and bathophenanthroline disulfonic acid and derivatized and analyzed by HPLC as previously described.[27,28] Malondialdehyde was measured by HPCL.[29] The protein content was measured by the Bradford method.[30]

DNA Isolation and Enzymatic Digestion

Cell DNA was isolated following the method of Gupta[31] with the modification described by Muñiz *et al.*[32] in which chloroform isoamyl alcohol (24:1) is used instead of phenol for the removal of proteins.[33]

Isolated DNA was washed twice with 70% ethanol, dried and dissolved in 200 µl of 10 mM Tris/HCl, 0.1 mM EDTA, 100 mM NaCl, (pH 7.0) for its enzymatic digestion, as previously described.[34] In brief, 0.5 µg DNA/µl was incubated with 100 units of DNase I in 40 µl Tris/HCl (10 mM and 10 µl) of 0.5 M $MgCl_2$ (the final concentration of 20 mM) at 37 °C for 1 h. The pH of the reaction mixture was then lowered with 15 µl of sodium acetate 0.5 M (pH 5.1); 10 µl of nuclease P1 (5 units) and 30 µl of 10 mM $ZnSO_4$ (to give a final concentration of 1 mM) were added, and mixture incubated for 1 h. After readjusting the pH with 100 µl of 0.4 M Tris/ClH (pH 7.8) followed by the addition of 20 µl alkaline phosphate (3 units), the samples were incubated for 30 min. Enzymes were precipitated with acetone (5 V), removed by centrifugation, and the supernatant evaporated to dryness.

8-OHdG Assay

The DNA hydrolysates were disolved in HPLC grade water and filtered through a 0.2-µm syringe filter before applying the samples to a Waters ODS HPLC column (2.5 × 0.46 i.d.; 5 µ particle size). The amount of 8-OHdG and dG in the DNA digest was measured by electrochemical and UV absorbance detection respectively, under the elution conditions previously described.[32] Standard samples of dG and 8-OHdG were analyzed to assure their good separation and to allow identification of those derived from cell DNA.

Analysis of H-2 Expression

Cells from B9 and G2 clones were incubated with the conditions described above with and

without 0.125 mM *t*-BOOH disolved in RPMI culture medium 4 h previously to H-2 expression analysis. Surface H-2 class I expression was determined with a standard method of indirect immunofluorescence. Cells (10^5) were washed twice in phosphate-buffered saline (PBS) and incubated for 30 min at 4 °C with the monoclonal antibodies (MoAb) anti-H-2K^dD^d (34.1.2), anti-H-2D^d (34.5.8) and anti-H-2L^d (24.14.8) obtained from the ATCC. Cells were washed twice in ice-cold PBS and incubated with a 1:40 dilution of FITC-labeled rabbit anti-mouse immunoglobulin (Cappel, West Chester, PA). After washing, fluorescence of cells was measured on a FACSort flow cytometer (Becton Dickinson, San Jose, CA). Ten thousand events were collected and analyzed.

NFκB Synthetic Oligonucleotides

Oligonucleotides containing the NFκB site[35] were synthesized with a System 200A DNA synthesizer (Beckman Instruments) and annealed. The duplex sequences are: TCGACAGAGGGACTTTCCGAGAGG and TTCCCTGAAAGGGTACTAG. This oligonucleotide probe was labelled by filling in both ends with α^{32}P dATP using Klenow fragment.

Cellular Extracts and Gel Mobility Shift Assay

Nuclear extracts in basal conditions and after incubation with 0.125 mM *t*-BOOH for 4 h and 1,000 U/ml γ-interferon (Amersham, Little Chalfont, UK) for 72 h, were prepared essentially as described by Dignam *et al.*[36] with minor modifications[37] from nuclei isolated from cells lysed in lysis buffer. The extracts were stored as aliquots at −80 °C. Protein concentration in nuclear extracts was determined with the BioRad (Richmond, CA) protein assay kit. A typical 20 µl reaction involved: 4 µg of nuclear protein, 1 µg of poly(dIdC) (Pharmacia, Uppsala, Sweden), 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol and 20,000 cpm probe (0.05 ng). In competition experiments 50 or 100 ng of double stranded "cold" competitor oligonucleotide was added to the reaction 5 min before the addition of the probe. All reactions were carried out at room temperature. Reactions were electrophoresed through 4% low ionic strength polyacrylamide gels (29:1), 0.4 × TBE (1 × TBE = 0.089 M Tris base, 0.089 M boric acid, 0.93 g/l $EDTANa_2$), fixed in 10% acetic acid, dried and exposed on XAR film (Kodak) at −80 °C.

Statistics

The statistical significance was assessed by Student's *t*-test.

RESULTS

Oxidative Stress-related Metabolites in Fibrosarcoma Derived Clones and BALB/c3T3 Fibroblasts

Three different cell types, two oncogenic, B9 and G2, and BALB/c3T3 normal fibroblasts were used in these experiments whose results are shown in Table I. There are significant differences comparing normal fibroblasts with G2 fibrosarcoma cell clone. Enzyme activities are significantly higher in G2 than in BALB/c3T3 fibroblasts (Table I). The percentage increases of catalase, SOD and GPx in G2 cell clone compared with control 3T3 cells were 141%, 54% and 38% respectively.

In B9 cell clone the antioxidant activities are similar to those of normal fibroblasts except for GPx which is significantly lower. There are also differences in the antioxidant enzyme activities between G2 and B9 cell clones. These are significantly lower in B9 cells.

Both reduced and oxidized glutathione are significantly higher in G2 cells than in normal fibroblasts or B9 cells (Table II). GSH concentration in G2 cells is two-fold higher than

TABLE I Comparative study of antioxidant enzymes in BALB/c3T3, G2, and B9 cells

Cell line	BALB/c3T3	G2	B9
Cat. (U/gProt.)	1.2 ± 0.5 (14)	2.9 ± 1.2 (20)*	1.3 ± 0.5 (10)$^{\#}$
SOD (U/mgProt.)	5.7 ± 1.7 (15)	8.8 ± 2.7 (10)*	6.3 ± 0.8 (9)$^{\#\#}$
GPx (U/gProt.)	5.0 ± 2.6 (16)	6.9 ± 1.2 (19)*	2.4 ± 1.1 (8)**,$^{\#}$

Results are mean ± S.D. with the number of experiments in parentheses. Assays were performed on individual cell culture plates and the mean of duplicate analysis were used for statistical calculations.
*$p < .005$ and **$p < .01$ comparing G2 and B9 with BALB/c3T3 cells.
$^{\#}p < .005$ and $^{\#\#}p < .05$ comparing G2 with B9 fibrosarcoma cells.

in BALB/c3T3 or B9 cells. The amount of GSSG in G2 cells is only 1.4-fold higher than in normal fibroblasts or B9 cells. Thus a lower GSSG/GSH ratio is calculated for the G2 cells in contrast with the values of 1.16 and 1.27 for 3T3 and B9 cells respectively.

The oxidation product 8-OHdG is higher in G2 and B9 clones as compared with BALB/c3T3 fibroblasts and there are no significant differences between the two tumorogenig cell lines. However, the degree of lipid peroxidation, as assessed by MDA levels, is higher in G2 cells than 3T3 or B9 cells.

Effect of t-BOOH on the Expression of MHC Class I Antigens in GR9 Fibrosarcoma Clones

The effect of t-BOOH on the expression of cell surface H-2 class I antigens on B9 and G2 clones was measured by fluorescence activated cell sorter (FACS) analysis using monoclonal antibodies (MoAbs) recognizing H-2D^{d}, H-2K^{d} and H-2L^{d} (Figure 1). A representative example of 8–10 experiments is shown. 0.125 mM t-BOOH induces the expression of H-2 class I antigens in both, G2 and B9 clones with differences worthwhile pointing out. As can be observed, in G2 clone treated with the xenobiotic, the percentage of fluorescent cells exposed to anti-H-2K^{d} and anti-H-2D^{d} antibodies is slightly higher than in control cells. However, the same clone increases the expression of the H-2L^{d} molecule from a basal value of 3–40% (92% increase) after 4 h incubation in the presence of t-BOOH.

In B9 clone cells, presenting under basal conditions, a lack in the expression of H-2K^{d} H-2D^{d} and H-2L^{d} class I molecules, the effect of t-BOOH is more evident and significant. In the presence of the oxidant, the increases of H-2K^{d}, H-2D^{d} and H-2L^{d} antigens were from 5% to 30%, from 15% to 46% and from 9% to 43%, representing 83%, 67% and 79% increase percentages respectively. It should be emphasized that the observed increase in the expression of H-2 class I antigens by t-BOOH runs and developes in the absence of apparent signs of cytotoxicity.

Effect of t-BOOH on the Activation of NFκB in B9 and G2 Clones

As shown in Figure 2, t-BOOH activates the retardation in native gels of the ^{32}P-labeled DNA probe encompassing the decameric NFκB

TABLE II Comparative study of GSH status and the levels of 8-OHdG and MDA in BALB/c3T3, G2 and B9 cells

Cell line	3T3	G2	B9
GSH (nmoles/gProt)	20.7 ± 5.33 (21)	46.2 ± 12.8 (7)***	19.4 ± 5.40 (10)$^{\#\#\#}$
GSSG (nmoles/gProt)	0.23 ± 0.08 (21)	0.33 ± 0.09 (7)***	0.25 ± 0.12 (10)$^{\#\#\#}$
(GSSG/GSH) × 100	1.16 ± 0.40 (21)	0.71 ± 0.20 (7)*	1.27 ± 0.49 (10)$^{\#\#\#}$
8-0HdG/10^{5} dG	2.14 ± 0.61 (7)	3.82 ± 1.63 (8)*	3.13 ± 0.75 (8)$^{\#}$
MDA nmol/gProt.	0.07 ± 0.006 (15)	0.25 ± 0.10 (16)***	0.06 ± 0.02 (21)$^{\#\#\#}$

Results are mean ± S.D. with the number of experiments in parentheses. Assays were performed on individual cell culture plates and the mean of duplicate analysis were used for statistical calculations. For 8-OHdG, each DNA sample was assayed twice by HPLC-EC detection as described in the Methods section.*$p < .05$, **$p < .01$ and ***$p < .005$ comparing values in BALB/c3T3 cells with G2 and B9 cells.
$^{\#}p < .05$, $^{\#\#}p < .01$ and $^{\#\#\#}p < .005$ comparing values in G2 with B9 cells.

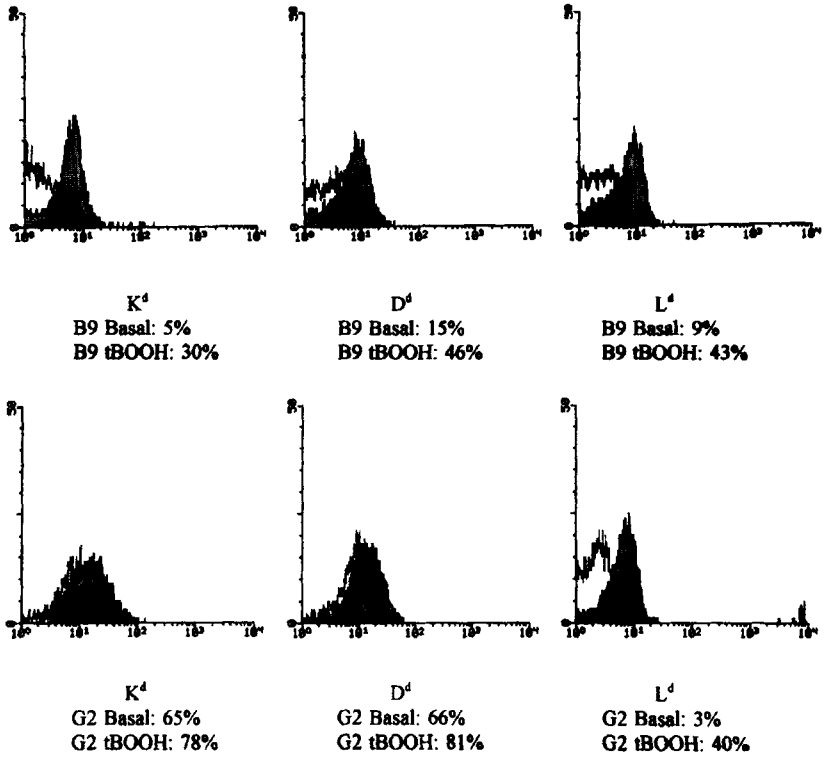

FIGURE 1 Effect of *t*-BOOH on MHC class I antigen expression in G2 and B9 murine fibrosarcoma clones. Fluorescence of cells was measured on a FACSort flow cytometer. Representative flow cytometry profiles show the effect of *t*-BOOH 0.125 mM (dark) by indirect immuno fluorescence of B9 (upper panel) and G2 (lower panel) cell clones with the following MoAbs, 34.1.2 (Anti H-2K^{d}D^d), 34.5.8 (Anti H-2D^d) and 28.14.8 (Anti H-2L^d). 10^3 events were collected and analyzed.

motif in both cell clones. The activation of NFκB was also shown by γ-INF in both cell clones although the incubation time required for this effect was 72 h which is in agreement with the previous observed lack time for H-2 antigen expression as will be discussed later.

DISCUSSION

The purpose of the present study was to evaluate the role of oxygen reactive species in the regulation of H-2 class I expression. We used *t*-BOOH because of its considerable propensity for generating free radicals and electrophilic reactive intermediates. Its intracelullar decomposition by cytochrome P450 leads to the generation of free radicals including $O_2^{\bullet -}$, $^{\bullet}OH$, peroxyl (LOO$^{\bullet}$) and alkoxyl (LO$^{\bullet}$) radicals. These latter radicals, peroxyl and alkoxyl, are common intermediates released from membrane phospholipids under oxidative stress conditions.[38] The interaction of these reactive products with other cell macromolecules such as protein receptors, transcription factors or nucleic acids has been established and in so doing signal transducing pathways may be modulated in both directions, enhancement or inhibition.[1,2,39]

Experiments were carried out using two cell clones (B9 and G2) derived from a well characterized fibrosarcoma.[21] The clones were selected based on their different biological properties, H-2 class I antigen expression under basal conditions, and tumorigenic potential.[22]

Analysis of oxidative stress parameters in both fibrosarcoma cell clones was performed

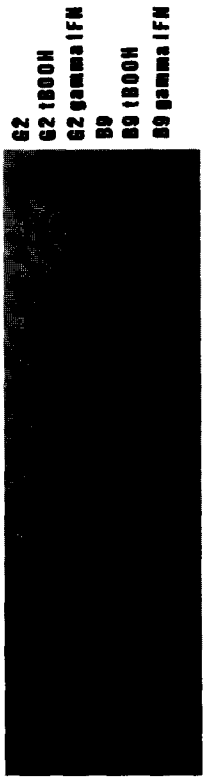

FIGURE 2 Effect of γ-INF and *t*-BOOH on the induction of κ-B binding activity in G2 and B9 cells. Nuclear extracts were prepared from untreated (control) or after 4 h and 72 h incubation in the presence of *t*-BOOH (0.125 mM) and γ-INF (1,000 U/ml) respectively. Electrophoretic mobility shift assays were done with 0.05 ng ^{32}P-labelled probe containing the NFκB consensus sequence. Competition experiments using 50–100 ng cold competitor together with other methodological details are specified in the corresponding epigraph.

and compared with those of BALB/c3T3 cells. There was a significant difference between the amount of antioxidant enzymes, especially between normal fibroblasts, where the respective activities were lower, and the G2 fibrosarcoma cell clone, presenting higher values of GSH, Cat, SOD and GPx. These differences may be attributed to a dispairment of oxygen metabolism in transformed cells[40,41] or to the special characteristics of the tumor cell line tested.[40,42]

The antioxidant efficiency of B9 and G2 were also different. Thus, G2-clone cells presented significant higher values of GSH, Cat., SOD and GPx when compared with B9 cells. In addition, GSH concentration in G2 cells was significantly (2-fold) higher when compared with the tripeptide levels in 3T3 and B9 cells. GSSG concentration in G2 cells was only 1.4-fold higher than that found in 3T3 or B9 cells. Therefore the redox status of G2 cells was more reduced showing a GSSG/GSH ratio of 0.71 in contrast with 1.16 and 1.27 obtained from 3T3 and B9 cells respectively. However, in spite of the higher GSH values in G2 cells which should represent a protection mechanisms against DNA damage and lipid peroxidation, as previously observed[32] these cells showed higher levels of 8-OHdG and MDA, which are considered parameters of choice in oxidative stress processes.[1,43,44]

We have shown that *t*-BOOH, increases the expression of H-2 class I antigens in both G2 and B9 fibrosarcoma cells, the effect being mediated by the activation of the transcription factor NFκB. Under basal conditions the expression of H-2K^d and H-2D^d is positive in G2 cells whereas in B9 cells it is negative.[21,22] These differences are difficult to explain through an oxidative stress mechanism. However, other molecular events such as differences in antigen processing and translocation, could explain the distinctive pattern of antigen expression observed between G2 and B9 cells under basal conditions.

H-2 increased expression by *t*-BOOH was especially observed in those antigens which were low or not expressed under basal conditions i.e. H-2L^d in G2 cells and H-2K^d, H-2D^d and H2-L^d in B9 cells. NFκB is a transcription factor which has specialized in the organism to induce the synthesis of defense and signalling proteins rapidly upon exposure of cells to a wide variety of most pathogenic agents, one of which is the xenobiotic used in this study.[45] Here we have shown that *t*-BOOH induces the activation of NFκB shown by electrophoretic mobility shift assay. However, it is likely that *t*-BOOH derived metabolites including $^{\bullet}$OH which easily diffuses

through the cell membranes, could directly activate NFκB by degrading or modifying IκB in the cytoplasmic P50-p65-IκB complex, a molecular mechanism allowing DNA-binding of NFκB and its translocation to the nucleus.[45] This hypothesis has been previously proposed and disccussed in the activation of NFκB by H_2O_2[45] or TNF-derived oxygen species.[46]

One of the most common characteristics of ROS effects is their rapid interactions with and modification of organic cell molecules. Consistant with this property, are also the short-timing effects of *t*-BOOH when compared with that of γ-INF, a well known stimulator of H-2 antigen expression and NFκB activation.[47] After 24 h of treatment γ-INF significantly induces H-2 class I antigen expression at the cell surface, with expression reaching a maximun after 72 h.[48] Using the same fibrosarcoma clones, grown and incubated under the incubation conditions reported previously, we have shown that *t*-BOOH enhances H-2 class I antigen expression and NFκB activation 4 h after treatment. Jointly considering these observations, it is concluded that *t*-BOOH is an efficient substrate in the induction of H-2 class I antigens. In agreement with previous reports[1,2,45,46] it is suggested that oxygen radicals acting as signal transducers or second messengers may integrate the diverse variety of NFκB-inducing effects, thus probably representing a common mechanism in the molecular machinery of gene expression. Recently the expression of MHC class II antigens by reactive oxygen species in peripheral blood dendritic cells has been reported[49] indicating that MHC antigens induction may respond to a common mechanism inplicating oxygen reactive species.

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