Immunology 1991 **72** 277–281 ADONIS 001928059100047H

Reduced stimulation of helper T cells by Ki-*ras* transformed cells

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Accepted for publication 22 October

SUMMARY

A number of viral genes and cellular oncogenes inhibit major histocompatibility complex (MHC) antigen expression at the cell surface. In the case of the inhibition of class I MHC antigens by viral genes this results in a reduced recognition by antigen-specific cytotoxic T cells. The activated Ki-*ras* cellular oncogene carried by the Ki-murine sarcoma virus (Ki-MuSV) in contrast inhibits class II MHC (or Ia) antigen expression on transformed cells. We have studied how transformation with Ki-*ras* affects recognition by alloreactive helper T cells. We found that the Ki-*ras* inhibition of class II MHC antigen expression led to greatly reduced stimulation of alloreactive T cells to proliferate and to secrete interferon-gamma (IFN-γ). These findings support our hypothesis that the ability of an oncogene to reduce class II MHC antigen expression is crucial to its ability to produce tumour cells.

INTRODUCTION

The helper T cells of the immune system are necessary to generate cytotoxic T cell, B cell and delayed-type hypersensitivity (DTH) responses to 'foreign' antigens. Normally such antigens are seen by helper T cells as complexes of class II major histocompatibility complex (MHC) molecules and peptides from the 'foreign' antigen—be it a viral antigen or an endogenous 'tumour antigen'.[1,2] This requirement for MHC molecules allows T-cell responses to be regulated by regulating the expression of MHC molecules themselves.[3] Although many cells express class I MHC antigens constitutively, they can be induced or regulated on most cells by a variety of cytokines, and particularly by the interferons (IFN).[4,5] Most cells, with the exception of B cells and dendritic cells, do not express class II MHC (or Ia) antigens constitutively but can be induced to do so with interferon-gamma (IFN-γ) either alone[6,7] or with tumour necrosis factor (TNF).[8] This allows for immune responses to be amplified upon infection since virus infection induces IFN-$\alpha\beta$ and responding T cells secrete IFN-γ.

Several viruses inhibit class I MHC antigen expression either directly or by inhibiting the response to IFN (reviewed by Maudsley *et al.*, 1989)[5] and this has been suggested to have a role in facilitating *in vivo* replication of the virus.[9] Indeed the inhibition of class I MHC antigen expression has been found to lead to reduced recognition of infected cells by allo-class I MHC antigen-specific cytotoxic T cells[9] or class I MHC antigen-restricted virus antigen-specific cytotoxic T cells.[10] Where the virus or viral gene responsible has been transforming, the reduction in class I MHC antigen expression has led to an increase in the ability of the cells to form tumours—presumably by enabling them to evade the class I MHC antigen-restricted arm of the immune system.[11] Several cellular oncogenes also inhibit class I MHC antigen expression on transfected cells[12–14] and, although this is thought to contribute to the ability of the cells to evade immune surveillance and hence to form tumours (as above)[11] there is as yet no evidence that this leads to reduced recognition by class I-restricted cytotoxic T cells.

We have previously demonstrated that cells infected with Kirsten Murine Sarcoma Virus (Ki-MuSV) in contrast express reduced levels of class II MHC antigen on their surfaces.[15,16] This specific effect is caused by the activated cellular oncogene Ki-*ras*-2[16] which has been acquired by Ki-MuSV and encodes the p21$^{Ki\text{-}ras}$ membrane-associated G protein.[17] It has also been found that cell surface expression of class II MHC antigens can be reduced by several distinct members of the *ras* oncogene family and in (at least) several different murine cell lines[15,16] (D. J. Maudsley and A. G. Morris, unpublished data). Activated Ki-*ras* appears to interfere with the signal transduction mechanisms that lead to increased class II MHC antigen expression but not, or to a much lesser degree, those signal transduction mechanisms leading to increased class I MHC antigen expression or to the antiviral response following IFN-γ binding to its receptor.[16,18]

These observations suggest a surprising role for *ras* oncogenes, which provides a means by which some transformed cells may evade immune surveillance. In further assessment of this we demonstrate in this report that Ki-*ras*-transformed cells display a reduced ability to stimulate allospecific T cells to proliferate and secrete IFN-γ.

MATERIALS AND METHODS

Mice

C3H/He and BALB/c mice were originally obtained from OLAC(UK) Ltd (Bicester, Oxon, U.K.) and bred locally. Six to 8-week-old males were used.

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Cells and culture conditions
The murine fibroblast line C3H $10T\frac{1}{2}$[19] is of C3H mouse origin and of the H-2^k haplotype. The activated Ki-*ras* ($Gly^{12}\rightarrow Ser^{12}$;$Ala^{59}\rightarrow Thr^{59}$; in a retroviral vector[20]) oncogene-transformed subline of C3H $10T\frac{1}{2}$, C3H 201, was derived as described elsewhere.[15] Cells were grown in G-MEM supplemented with 10% foetal calf serum and 4 mM L-glutamine.

IFN-γ and MHC antigen induction
Recombinant murine IFN-γ (rMuIFN-γ) was produced in CHO cells and purified to a specific activity of 10^6 U/ml, as described elsewhere.[21] Fibroblasts were treated with 100 U/ml IFN-γ and harvested 4 days later for staining and using as stimulators for T-cell responses.

T-cell proliferation
Alloresponsive T cells were prepared as described elsewhere.[22] Briefly, BALB/c mice were immunized i.p. with C3H/He spleen cells. Approximately 7 days later spleens were harvested and restimulated *in vitro* with mitomycin C-treated C3H/He spleen cells. Seven days later viable cells were separated by density. All stimulator cells were treated with mitomycin C (25 μg/ml for 30 min at 37°) to prevent proliferation. Cells were cultured in triplicate in 96-well round-bottomed microtitre plates at 10^4 stimulator cells and 3×10^4 responder cells per well. Two μCi [^{3}H]thymidine were added to each well after 24 h and cells harvested at 64 hr. Data are expressed as mean ± SE of c.p.m. [^{3}H]thymidine incorporation. Significance was determined using Student's *t*-test.

T-cell IFN-γ secretion
Again alloresponsive T cells were prepared by immunizing BALB/c mice with C3H/He spleen cells.[22] Stimulators were C3H/He spleen cells, C3H $10T\frac{1}{2}$ and Ki-*ras* transformed C3H $10T\frac{1}{2}$. C3H $10T\frac{1}{2}$ and Ki-*ras* C3H $10T\frac{1}{2}$ were incubated with or without 100 U/ml IFN-γ 4 days prior to use. Co-culture of responding T cells and stimulator cells was essentially as for T-cell proliferation except that 12-well Costar plates were used. 10^6 cells/ml responders were used throughout with or without stimulator cells at 5×10^4/ml (fibroblasts) or 5×10^5/ml (C3H/He spleen cells). Supernatants of the cultures were harvested 2 days after stimulation and assayed as described elsewhere.[23]

Monoclonal antibodies and flow cytofluorometry
Cells were harvested and stained as described previously.[15] Monoclonal antibodies (mAb) used against MHC antigens were as follows: anti-H-$2K^k$, TIB95/11.4.1; anti-H-$2A^k$, TIB92; anti-H-$2E^k$, HB32; anti-H-$2A^d$, HB3 (negative control). All mAb were used at saturating concentrations. All hybridoma cell lines are available from the American Type Culture Collection, Rockville, MA. FITC-goat anti-mouse Ig (Cappel Laboratories, Malvern, PA) was used as the second layer, again at saturating concentration (1:60 in RPMI-1640, HEPES-buffered, supplemented with 5% foetal calf serum and 0·2% sodium azide). Cells were analysed on a FACStar flow cytometer (Becton-Dickinson & Co., Mountain View, CA). Using the Consort 30 computer program, cells were carefully gated on forward scatter and side scatter parameters to exclude cell doublets and cell debris and to include only single whole cells. Within a single experiment the same gates were used for all cell types. The Consort 30 computer program was then used to obtain fluorescence histograms and mean cell fluorescence intensities.

Tumourigenicity of cells
Cells were harvested and washed in medium. A single cell suspension was made. The appropriate number of cells were suspended in 100 μl PBS and injected subcutaneously in the flank. The mice were regularly inspected for tumour growth for 6 weeks.

RESULTS

MHC antigen induction

C3H $10T\frac{1}{2}$ fibroblasts and Ki-*ras*-transformed C3H $10T\frac{1}{2}$ cells were cultured with and without 100 U/ml IFN-γ for use as stimulator cells. After 4 days the cells were harvested and assayed for class I (H-$2K^k$) and class II (H-$2A^k$and H-$2E^k$) MHC antigen expression (Fig. 1). The Ki-*ras*-transformed cells responded normally to IFN-γ with respect to class I MHC antigen expression in that H-$2K^k$ was clearly expressed, but failed to express significant amounts of class II MHC antigens (I-A^k or I-E^k). Hence transfection of C3H $10T\frac{1}{2}$ cells (H-2^k) with activated Ki-*ras* ($Gly^{12}\rightarrow Ser^{12}$;$Ala^{59}\rightarrow Thr^{59}$; in a retroviral vector[20]) oncogene resulted in loss of ability of the cells to respond to IFN-γ with class II MHC antigen expression (Fig. 1).

Alloproliferation

To determine whether this change in class II inducibility enables the cells to evade helper T cell responses the above cells were used as stimulator cells for an alloreactive (BALB/c [H-2^d] anti-C3H/He[H-2^k]) T-cell line (Fig. 2). C3H $10T\frac{1}{2}$ cells not treated with IFN-γ expressed no class II antigens, as determined by indirect immunofluorescence and flow cytometry (Fig. 1), and failed to stimulate allogeneic T cells to proliferate (Fig. 2). In fact background proliferation of the responding cell population was inhibited by these cells. IFN-γ treatment of C3H $10T\frac{1}{2}$ resulted in class II (Ia) antigen expression on the cells (Fig. 1) and, when used as stimulator cells, in restored proliferation that was significantly greater than for either the unstimulated responder population or C3H $10T\frac{1}{2}$-stimulated responders (Fig. 2, $P<0.05$ on both accounts). Stimulation of responding T cells with C3H/He (H-2^k) spleen cells also resulted in clear stimulation of proliferation (Fig. 2). Ki-*ras*-transformed cells, with or without treatment with IFN-γ, were used as stimulators. Again cells not treated with IFN-γ inhibited the background proliferation of unstimulated responding T cells. This inhibition was prevented by IFN-γ treatment of the stimulator cells (Fig. 2) but no additional proliferation was seen above the background level of proliferation of unstimulated responder T cells. The IFN-γ-treated Ki-*ras* C3H $10T\frac{1}{2}$ cells expressed high levels of class I MHC antigens, thus demonstrting that the allospecific T-cell proliferation response was class II not class I restricted. However, some, but very low, amounts of class II MHC antigens (Fig. 1) in addition to the class I antigens were expressed on these cells. The possibility remains therefore that these MHC antigens might have stimulated at least some proliferation rather than IFN-γ treatment removing the inhibition by fibroblasts. Even so it is clear that Ki-*ras*-transformed IFN-γ-treated C3H $10T\frac{1}{2}$ cells stimulate allospecific T cells to

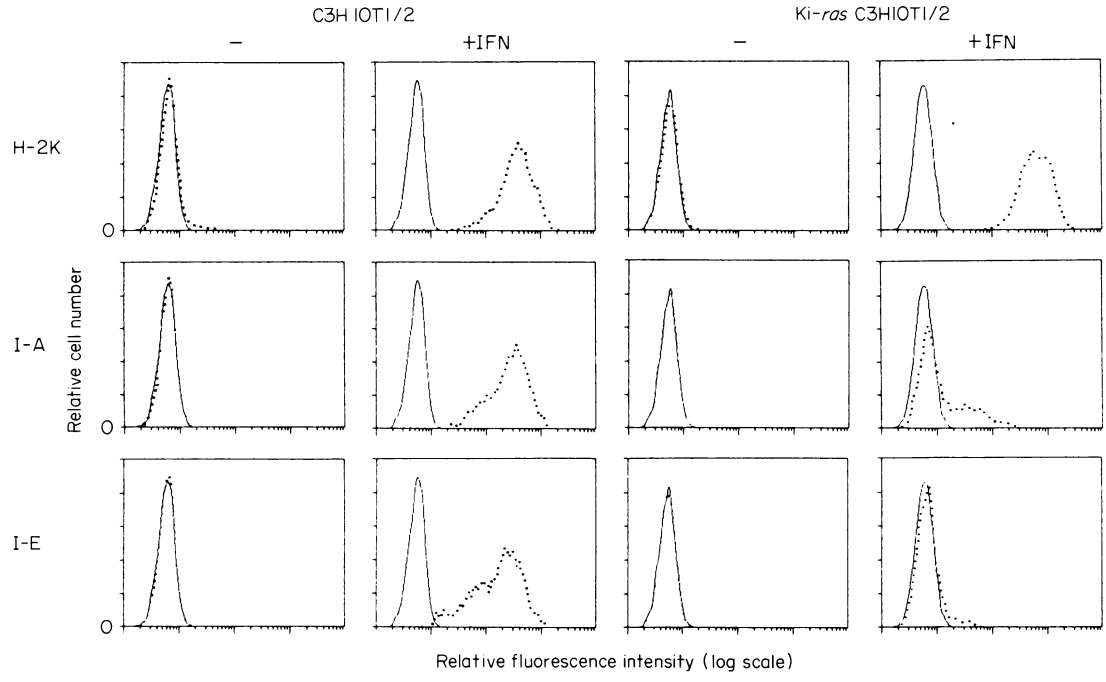

Figure 1. The induction by IFN-γ of MHC antigens (H-2K, I-A^k, I-E^k) on untransformed and Ki-*ras* transformed C3H 10T$\frac{1}{2}$ fibroblasts used as stimulator cells for T-cell proliferation. Solid lines: I-A^d control; dotted lines: specific staining with monoclonal antibodies (top row) to H-$2K^k$ (middle row), to I-A^k, and (bottom row) to I-E^k. First column: untreated C3H 10T$\frac{1}{2}$ fibroblasts; second column: IFN-γ-treated C3H 10T$\frac{1}{2}$ cells; third column: untreated Ki-*ras*-transformed C3H 10T$\frac{1}{2}$ cells; fourth column: IFN-γ-treated Ki-*ras* transformed C3H 10T$\frac{1}{2}$.

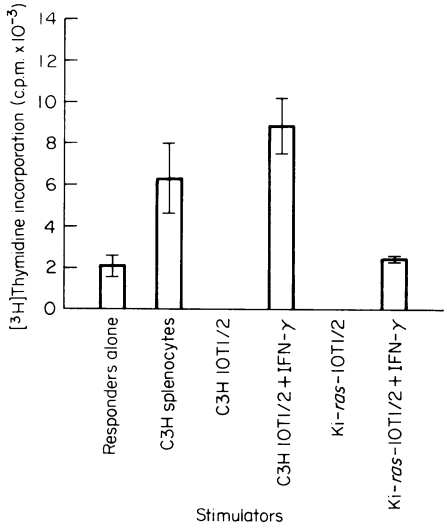

Figure 2. Allospecific (BALB/c; H-2^d) T cells proliferate in response to class II MHC antigens on C3H/He (H-2^k) spleen cells or IFN-γ-treated C3H 10T$\frac{1}{2}$ fibroblasts but not to class II-deficient IFN-γ-treated Ki-*ras* transformed fibroblasts.

Table 1. Stimulation of IFN-γ secretion by untransformed and activated Ki-*ras* transformed fibroblasts

Responders*	Stimulators	Treatment of stimulators†	IFN-γ (U ml)
+	—	—	40
+	C3H/He Spleen	—	1000
+	C3H 10T$\frac{1}{2}$	—	60
+	C3H 10T$\frac{1}{2}$	+	400
+	Ki-*ras* C3H 10T$\frac{1}{2}$	—	80
+	Ki-*ras* C3H 10T$\frac{1}{2}$	+	80

* BALB/c (H-2^d) anti-C3H/He (H-2^k) at 10^6 cells/ml.

† C3H 10T$\frac{1}{2}$ and Ki-*ras* C3H 10T$\frac{1}{2}$ were incubated with or without 100 U/ml IFN-γ 4 days prior to use (Fig. 1). Supernatants of the cultures were harvested 2 days after stimulation and assayed for IFN-γ.

proliferate substantially less (if at all) than untransformed IFN-γ-treated C3H 10T$\frac{1}{2}$ cells.

The fivefold stimulation of responding T cells is smaller than expected. The data are, however, statistically significant (that is, for the differences between the IFN-γ-treated C3H 10T$\frac{1}{2}$ cell-stimulated response and either unstimulated proliferation or IFN-γ-treated Ki-*ras*-transformed C3H 10T$\frac{1}{2}$-stimulated proliferation, $P < 0{\cdot}05$) and the same trend of responses was seen in three further experiments. The low stimulation may reflect two factors. Firstly these fibroblasts are intrinsically inhibitory of T-cell proliferation, as shown by the inhibition of background proliferation by untreated fibroblasts (i.e. by cells not expressing class II MHC antigens). Secondly class II MHC antigen expression alone is not necessarily sufficient for efficient T-cell stimulation.[24] In the experiment shown the stimulation by H-2^k spleen cells is also relatively low (although this may reflect 5×10^4 cell/ml being optimal for stimulation by fibroblasts but not for spleen cells).

In conclusion, the data clearly show (Fig. 2) that stimulation of allospecific T cells only occurs where the stimulators are

expressing significant amounts of class II MHC antigens and that the residual amounts expressed on Ki-*ras*-transformed fibroblasts are insufficient to stimulate T-cell responses.

Stimulation of IFN-γ production

C3H 10T½ fibroblasts and Ki-*ras* C3H 10T½ were incubated with or without 100 U/ml IFN-γ 4 days prior to use as stimulator cells (Fig. 1). Alloreactive (BALB/c [H-2^d] anti-C3H/He [H-2^k]) T cells were cultured with or without these stimulator cells or C3H/He spleen cells essentially as above for T-cell proliferation (Fig. 2), except that 12-well Costar plates were used. Supernatants of the cultures were harvested 2 days after stimulation and assayed for IFN-γ. Table 1 shows that C3H/He spleen cells and IFN-γ treated C3H 10T½ cells stimulated IFN-γ secretion. In contrast Ki-*ras*-transformed cells (IFN-γ treated or not) and untreated C3H 10T½ did not.

Again the failure of IFN-γ-treated Ki-*ras* transformed C3H 10T½ cells to stimulate T cells demonstrates that the helper T-cell response was class II and not class I restricted. It also demonstrates that the residual levels of class II MHC antigen expression on IFN-γ-treated Ki-*ras* tranformed C3H 10T½ cells were not sufficient to stimulate the T cells to secrete IFN-γ.

Tumourigenicity

Three × 10^5 Ki-*ras*-transformed C3H 10T½ cells injected subcutaneously produced tumours in 100% (38/38) of syngeneic C3H/He mice within 14 days, whereas 3 × 10^6 parental cells failed to produce any tumours (0/10) within 6 weeks.

DISCUSSION

The class II-restricted helper T-cell response to foreign or tumour antigen is dependent on the concentration of the class II MHC antigen expression.[3,25-27] We have previously shown that Ki-*ras* inhibits expression of class II on transformed cells. The possible significance for tumour immunology of this finding would ideally be studied using tumour antigen-specific, class II-restricted helper T cells. No such tumour antigen has been described for C3H 10T½ cells. Alloantigen-specific (class II MHC antigen specific) helper T-cell responses are analogous to tumour antigen-specific responses[28] in that in both cases the T cells recognize a class II MHC antigen-peptide antigen complex and recognition is normally by the α/β T-cell receptor in association with the CD4 T-cell surface molecule. Because of this we chose to study how T cells would recognize class II antigens on C3H 10T½ cells transformed with Ki-*ras*.

The T cells used responded to IFN-γ-treated C3H 10T½ cells, which were expressing class II MHC antigens, but not to IFN-γ-treated Ki-*ras*-transformed C3H 10T½ cells, which were expressing little or no class II MHC antigen. This was the case both for T-cell proliferative responses and for T cell IFN-γ-secretion responses. Hence the level of class II antigen expression by the tumour cells affects their ability to simulate T cells to proliferate and secrete lymphokines (at least IFN-γ). The data also tell us that the residual expression of Ia antigens on IFN-γ-treated Ki-*ras* C3H 10T½ is insufficient to stimulate helper T cells to proliferate and secrete IFN-γ under the conditions used. By implication this alteration in the immunogenicity of tumour cells will affect, it is predicted, their tumourigenicity. In agreement with this prediction, Ki-*ras*-transformed C3H 10T½ cells produced tumours in syngeneic C3H/He mice, whereas C3H 10T½ cells did not, although this may be due in part to non-immune mechanisms, and to other properties of Ki-*ras*-transformed cells.

Analogous to this there is clear evidence that decreased *class I* MHC antigen expression affects the tumourigenicity of transformed cells, presumably by making them less visible to CD8$^+$ cytotoxic T cells.[5,29,30] An equally important role for *class II* MHC antigens in immune resistance to tumours by CD4$^+$ T cells is suggested indirectly by several other reports. Smith *et al.*[31] for example report that in human colorectal cancers (where, interestingly, *ras* activation is common)[32,33] tumour cells fail to express class II MHC antigens whereas expression on adjacent normal cells is high. This and other human evidence is supported by indirect evidence from a number of animal systems where class II expression correlates with tumour regression rather than progression[34] and either deletion of CD4$^+$ T cells[35,36] or neutralization of IFN-γ[35] abrogates immunity to tumours *in vivo*. In support of these indirect *in vivo* findings the report here demonstrates that Ki-*ras*-inhibited class II antigen expression results in an abolition of or reduction in the ability of tumour cells to stimulate T cells *in vitro*, i.e. an evasion by the tumour cells of T-cell immunity.

It is surprising that oncogenes should enable tumour cells to evade immune surveillance in such a straightforward way—by down-regulating MHC antigen expression. However, activated Ki-*ras* is not unique; activated Ha-*ras* (both human and rat) also inhibits class II MHC antigen expression (D. J. Maudsley and A. G. Morris, unpublished observations), and *myc* and N-*myc* down-regulate class I MHC antigen expression in some cell types.[12-14] Inhibition of class I MHC and class II MHC antigen expression by oncogenes may therefore be widespread phenomena and may make a significant contribution towards tumourigenesis.

The abolition, by Ki-*ras* transformation, of the stimulation of helper T-cell IFN-γ secretion (Table 1) may have particular significance in a number of ways. Firstly, while class II antigens are required for stimulating CD4$^+$ helper T cells,[25] the IFN-γ secreted on stimulation is probably the main inducer of further class II expression. This is likely to act as a positive feedback loop naturally amplifying T-cell responses.[5] Secondly, IFN-γ is secreted by Th1 (and not Th2) cells.[37] These also have the property of secreting IL-2 (required for cytotoxic T-cell responses), and are responsible for delayed-type hypersensitivity[38] (largely mediated via IFN-γ itself).[39] This is probably important in attracting 'infiltrating lymphocytes' to tumours. Thirdly, IFN-γ is responsible for macrophage activation. We propose that the down-regulation of class II antigens by Ki-*ras* transformation, in particular interrupts the positive feedback loop involving IFN-γ allowing the tumour cells to escape immune surveillance.

Thus we provide direct evidence for a link between oncogene activation and evasion of immune responses, via inhibition of MHC antigen expression.

ACKNOWLEDGMENTS

We thank Dr S. Maudsley for comments on the manuscript. We thank the Cancer Research Campaign for generous support for this work.

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