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This study aims to genetically modify the tumor cells and use them to induce efficient and vigorous anti-tumor immunity. Murine mammary carcinoma cell lines, 66.1 (metastatic and non-immunogenic) and 410.4 (non-metastatic and immunogenic) are used in the initial studies. To investigate whether or not costimulation of $CD8^+$ T cells is sufficient to induce tumor rejection, the wild type 66.1 tumor cells (66.1/WT) were transfected with B7.I cDNA and stable lines and clones that constitutively express B7.I protein (66.1/B7.I^+) were generated. Subcutaneous injection of live 66.1/B7.I^+ tumor cells into Balb/c mice showed significant delay in the growth compared to 66.1/WT tumor cells. However, complete rejection was not seen, indicating that activation of $CD8^+$ T cells alone is not sufficient to induce tumor rejection. Further studies are under way wherein transfectants expressing both B7.I and class II MHC molecules will be used to induce tumor immunity in the syngenic host. These trasfectants will help to understand the role of $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells in the induction of anti-tumor immunity.

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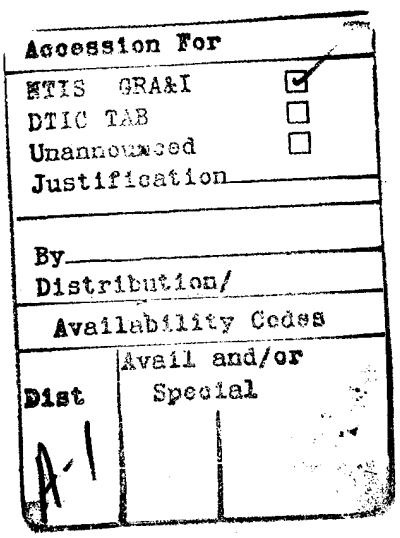

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(5) INTRODUCTION:

Immunotherapy of cancer relies on the fact that the tumor cells escape from the immunesurveilance mechanisms of the host because of the inadequate recognition by and activation of tumor-specific T lymphocytes, rather than the absence of tumor-specific antigen. Numerous studies have shown the presence of specific tumor associated antigens (1), although certain tumor antigens are expressed by the malignant tumor cells as well as their normal counter parts (2). Nevertheless, enhancing (tumor) antigen-specific response will help control malignant growth of tumor cells and induce anti-tumor immunity. Initial trials of immunotherapy of cancer in general, breast cancer in particular, used rather non-specific immunostimulators ranging from Bacillus Calmette-Guerin (BCG) and levamisole to interferon, interleukins and monoclonal antibodies (mAb) (3). The present proposal aims to investigate an alternate strategy to enhance anti-tumor immunity by facilitating the recognition of tumor antigens and inducing a vigorous tumor-specific T cell response in the syngenic host.

As known with nominal antigens, it is reasonable to speculate that anti-tumor immune response will involve recognition of the "tumor antigen" by $CD4^+$ helper T cells (Th) and $CD8^+$ cytotoxic T cells (Tc or CTL) in the context of MHC class II and class I molecules, respectively (4). Clonal expansion of these T cells and their subsequent functional maturation is governed by cytokines and other accessory molecules present on APC (5). Some of these cytokines besides being autocrine growth factors, also enhance the cytotoxicity potency of the effector cells (6, 7).

A variety of cytokines including IL-2, IL-4, IL-10, IL-12, TNFa, IFNg and GM-CSF have been used to augment tumor regression *in vivo* (8-17). In some cases the therapeutically effective concentrations of cytokines are accompanied by toxic side effects. This problem was overcome by cytokine gene therapy, in which tumor cells were transfected with cytokine genes and sufficient amount of cytokine was released at the tumor site with out leading to high systemic levels (8, 12-16). However, the anti-tumor immunity induced was relatively short-lived and the efficacy varied depending on the tumor model under study (18). In a recent report on a murine mammary tumor, IL-2 transfected 4T07 (4T07-IL-2) cells partially protected autologous host against wild type 4T07 tumor challenge, but failed to protect against a metastatic variant (4T1) derived from the same parent line (19). In another study, IL-10 transfection inhibited metastasis of 410.4 and 66.1 mammary carcinnoma tumor cells, but did not prevent growth when injected subcutaneously (20).

Tumor cells as APC:

While $CD8^+$ T cells are the major effector cells in killing the target tumor cells, their proliferation and functional maturation requires help from $CD4^+$ T cells. Since MHC class II gene expression is tightly regulated and restricted to professional APCs, majority of the tumor cells do not express MHC class II proteins and can not stimulate $CD4^+$ T cells. As a consequence, in the absence of $CD4^+$ T cell help, the $CD8^+$ T cells are not sufficiently matured or activated. We and others have shown that constitutive expression of MHC class II genes in tumor cells resulted in rejection of the tumor cells by sygenic host (21-24). Rejection of class II^+ tranfectants resulted in the induction of protective immunity against wild type tumor cells (21-24). These findings strongly suggest that constitutive expression of MHC class II molecules on tumor cells **converts the tumor cells into APC** and enable them to directly present the tumor peptides to $CD4^+$ tumor-specific Th cells leading to potent anti-tumor immunity (25).

Costimulatory/accessory signals in anti-tumor immunity:

Maximal activation of T cells (both Th and Tc) has been shown to require an antigen-specific signal provided by the engagement of the T cell receptor (TCR) and MHC/peptide complex and an antigen-independent second signal provided by the interaction between a number of costimulatory molecules and their corresponding receptors on the responding T cell (26-29). Amongst a variety of molecules involved in costimulation, the interaction between B7 family of proteins on APC and their ligands, CD28 and CTLA-4 on T cells has been well documented (30-34). We and others have shown that constitutive expression of B7-1 and B7-2 molecules on tumor cells resulted in the rejection of these gene-modified tumor cells and prior injection of $B7^+$ tumors induced protective immunity against subsequent wild type tumor challenges in syngenic hosts (35-37). In addition, a synergistic effect was observed when tumor cells were gene modified to express both MHC class II and B7-1 molecules; these cells are potent vaccine capable of inducing complete regression of previously established tumors (38).

Based on these results, the present study is to investigate the effect of constitutive expression of MHC class II and costimulatory molecules, B7-1 and B7-2 in enhancing anti-tumor immunity against murine mammary carcinoma as a model for human breast cancer.

Tumor Model:

Originally, it was proposed to use the mammary tumor cell lines, 4T07 and 4T1, which are derived from a parental spontaneous tumor in Balb/c mice. While the

subline 4T07 is very weekly immunogenic and does not spontaneously metastasize (although experimental metastasis has been observed with this subline), the subline 4T1 is virtually non-immunogenic and highly (spontaneously) metastatic (39, 40). They both do not express MHC class II molecules (Tsai, personnel communication). These criteria bring these two tumor cell lines closer to human breast cancer and offer a better model system to study. However, the studies in this report have been performed with two other cell lines, 410.4 and 66.1, which are derived from the same parental tumor cell line and satisfy all the criteria of 4T07 and 4T1, respectively.

Tumor cell lines 410.4 and 66.1 will be transfected with plasmid vectors containing cDNA encoding murine B7-1, B7-2 proteins and/or cDNA encoding syngenic MHC class II alpha and beta polypeptides forming I-A^d molecule. To allow selection of transfectants from the wild type tumor cells, plasmid vectors containing neomycin or hygromycin resistant gene will be cotransfected. Transfection will be performed using Lipofectine or Lipofectamine as previously described (21, 37). Transfectants will be grown in medium containing appropriate selection drug at a predetermined concentration. Surface expression of B7-1, B7-2 and I-A^d proteins will be monitored by flow cytometry analysis using appropriate monoclonal antibodies. Stably transfected tumor cell lines will be established, and clones will be generated by limiting dilution. Cloned transfected tumor cell lines will be periodically monitored for the surface expression of the protein(s). The tumorigenicity of the transfected tumor cells will be determined by in vivo tumor challenge experiments.

(6) BODY: Experimental Results

A murine mammary carcinoma was used in this study as a model for human breast cancer. In the initial experiments, two sublines of a spontaneous Balb/c -derived tumor were used. The subline 410.4 is weakly immunogenic and does not metastasize spontaneously. Another subline 66.1 is non-immunogenic and spontaneously metastasize in the syngenic host, Balb/c mice. These characteristics of the 410.4 and 66.1 sublines satisfied their use as substitutes for the originally proposed sublines 4T07 and 4T1 having the same characteristics.

<u>In vivo growth kinetics</u>:

In order to determine the growth pattern of the wild type 66.1 and 410.4 tumor cells (66.1/WT, 410.4/WT) naive Balb/c mice were injected subcutaneously (s.c.) in the

flank with different doses of 66.1 and 410.4 tumor cells. The tumor growth was monitored as described above. Figure 1 depicts the results of the experiment. After an initial lag period of 10 - 20 days 66.1/WT tumor cells progressively grew in the naive Balb/c mice. By day 50 - 60 most of the tumors reached the size of about 15 - 20 mm in diameter (Fig. 1A-C) and often ulcerated, at which point the mice were sacrificed by euthanasia. The subline 410.4 grew relatively slow (Fig.1D).

In the following experiments, the subline 66.1 was used and similar experiments will be carried out using the subline 410.4 as well.

Drug sensitivity and tolerance:

As a prerequisite for the transfection experiments the sensitivity of the 66.1/WT tumor cells to the selection drugs, G418 and Hygromycin, was determined. Two approaches were taken. First, the 66.1/WT tumor cells were cultured in the absence or presence of various concentrations of G418 and Hygromycin. Cell viability was monitored daily by examination under the microscope. A progressive cell death was observed with icreased concentration of both the drugs from day 2 of culture, and by day 4-5 almost all cells were killed at the minimal concentration of 400 μg/ml G418 and 200 μg/ml Hygromycin.

To determine the drug sensitivity in a non-subjective way, a second approach was undertaken. 66.1/WT tumor cells were cultured at two different concentrations in the absence and presence of various concentrations of G418 and Hygromycin in 96 well plates. Cell viability and proliferation was measured by determining the uptake of ^{3}H-thymidine (cpm) during the last 18 hr of the three day cultures. Values below 1000 cpm are considered background. G418 at 400 μg/ml resulted in maximum inhibition of cell proliferation at 100 cells/well and at higher cell number (1000 cell/well) a plateu was seen between 300 and 500 μg/ml (Fig. 2A). Hygromycin at 200 μg/ml caused significant inhibition of cell proliferation particularly at lower cell number and further increase in the amount of drug did not result significant difference (Fig. 2B).

Based on these experiments it was decided to use 400 μg/ml G418 and 200 μg/ml Hygromycin for the transfection experiments. At these drug concentrations virtually none of the tumor cells grew even after prolonged culturing. This indicated that the background ^{3}H-thymidine uptake is not due to slow growth of a small number of drug resistant cells.

Constitutive expression of B7-1 on 66.1/WT tumor cells:

66.1/WT tumor cells express endogenously MHC class I molecules K^d and D^d, but do not express T cell costimulatory molecule B7-I. It is hypothesized that the failure of 661./WT tumor cells to induce effective anti-tumor immunity is due to lack of costimulation of $CD8^+$ T cells in the host which could potentially recognize the "tumor antigen" in the context of MHC class I molecules. To test this hypothesis 66.1/WT tumor cells are transfected with cDNA encoding murine B7-I molecule. The drug (G418) resistant cells were grown as lines and the different lines of transfectants were screened for the stable expression of B7-I protein. Figure 3 depicts the flowcytometer analysis of 66.1/B7-I^+ lines. The level of expression of the transfected gene product (B7-I) is comparable to the expression of endogenous K^d molecule (Fig. 3A, B). Some of these lines have been cloned by limiting dilution and tested for the stable expression of B7-I molecule.

Immunogenicity of 66.1/B7-I^+ tumor cells:

To test the effect of constitutive expression of B7-I on tumor rejection, groups of Balb/c mice were s.c. injected with different 66.1/B7-I^+ lines. The control group of mice received the same number of 66.1/WT tumor cells. As observed before, the 66.1/WT tumor cells grew progressively with a lag period of about 15-20 days. By contrast, the 66.1/B7-I^+ lines showed a significant delay in the growth, although different 66.1/B7.I^+ lines showed variation in their in vivo growth kinetics. Measurable tumor was not seen until about 40 - 60 days after inoculation and in some cases the maximum tumor size was significantly less than obtained with the inoculation of equal number of 66.1/WT tumor cells (Fig. 4 A-D). The 66.1/WT and 66.1/B7-I^+ tumor cells did not exhibit any difference in their growth in vitro. Further experiments using immunocompromised mice are underway to establish that the retarded in vivo growth of 66.1/B7-I^+ tumor cells is a result of a possible immune activation, albeit not strong enough to cause complete rejection.

To further understand the immunogenicity of 66.1/B7-I^+ tumor cells, experiments have been initiated to test whether prior injection of 66.1/B7-I^+ tumor cells will be able to induce protective immunity against subsequent challenge with 66.1/WT tumor cells. To avoid the initial growth of the "potentially immunizing" 66.1/B7-I^+ tumor cells they need to be growth arrested. This possibility is being tested as described below.

Irradiated 66.1/WT tumor cells do not induce immunity:

It was a prerequisite to know whether or not irradiated unmodified 66.1/WT tumor cells will be able to induce immunity to protect subsequent challenge with live 66.1/WT tumor cells. Accordingly, the 66.1/WT tumor cells were subjected to various dosages of radiation and cultured at different cell densities. The effect of radiation on the in vitro growth was monitored as described above for drug sensitivity. A radiation dose-dependent increase in the growth arrest in vitro was observed (Fig. 5). When Balb/c mice were injected s.c. with irradiated 66.1/WT tumor cells no tumor growth was observed up to 80 days. However, when these mice were subsequently challenged with live 66.1/WT tumor cells all the mice developed malignant tumor indicating that prior inoculation of irradiated 66.1/WT tumor cells did not induce any significant immunity (Fig. 6A, B).

Can irradiated 66.1/B7-I$^+$ tumor cells induce protective immunity ?

We and others, using different tumor models, have shown that irradiated, gene-modified tumor cells are able to induce protective immunity in the host against subsequent challenge with wild type, unmodified live tumor cells. Therefore, it was of interest to investigate whether irradiated 66.1/B7-I$^+$ tumor cells will be able to induce protective immunity. This is an ongoing experiment. Groups of Balb/c mice received irradiated 66.1/B7-I$^+$ or irradiated 66.1/WT (control group) tumor cells. Eighty days later all mice have been challenged with live 66.1/WT tumor cells. The final outcome of this experiment is yet to be known.

Constitutive expression of MHC class II on 66.1/WT tumor cells:

The 66.1/B7-I$^+$ tumor cells could potentially costimulate CD8$^+$ effector T cells. However, the failure to see the complete rejection of 66.1/B7-I$^+$ tumor cells could be due to lack of T cell help, in the form of cytokines secreted by CD4$^+$ T cells. To facilitate the activation of tumor-specific CD4$^+$ T cells, 66.1/WT tumor cells have been transfected with cDNA encoding the alpha and beta chains of syngenic MHC class II (I-A^d) molecule. The drug resistent cells are growing and are being screened for the surface expression of I-A^d molecule. When stable lines and clones expressing I-A^d molecule become available they will be used in the in vivo tumor challenge and protection experiments.

(7) CONCLUSIONS:

The in vivo growth kinetics of 66.1 cell line in the syngenic host, Balb/c mice was established with different doses of inoculum. This will be used to compare the growth rates of transfectants. The growth kinetics of the wild type tumor will be used in the later experiments which will address the therapeutic efficacy of immunogenic transfectants. The growth kinetics of the variant 410.4 line has been done at a single inoculum dose and this has to elaborated with different doses to allow paralell comparision with 66.1 tumor cells.

The drug sensitivity and tolerance limits for the drugs G418 and Hygromycin has been studied with the 66.1 cell line. This has been used to determine the optimal drug concentration for selection of appropriate transfectants. Similar testing has to be done with the 410.4 cell line.

The first question that has been addressed is whether or not B7-I transfectants can costimulate MHC class I restricted $CD8^+$ T cells sufficiently enough to induce tumor rejection. Stable 66.1/B7-1^+ lines have shown significantly retarded growth in vivo compared to the 66.1/WT tumor cells. This is being substantiated by using the clones derived from 66.1/B7-1^+ lines. Additional experiments will be performed to compare the in vivo growth of 66.1 and 66.1/B7-1^+ clones using nude mice. Since the costimulatory molecules B7-1 and B7-2 have been shown to have different effects in different tumor models, similar experiments will be performed using B7-2 transfectants. A recent report from another laboratory has shown that IL-10 transfected 410.4 and 66.1 tumor cells grow when injected subcutaneously like the wild type cells, but unlike wild type cells, IL-10 transfectants do not metastasize. It will be interesting to know whether or not 66.1/B7-1^+ transfectants can metastasize.

Prior injection of irradiated 66.1/WT tumor cells did not confer any protective immunity against a subsequent challenge with live wild type 66.1 tumor cells indicating their inability to activate $CD8^+$ T cells. It will be of interest to see whether irradiated 66.1/B7-1^+ tumor cells will, by virtue of costimulation, be able to induce protective immunity against challenge with live 66.1 tumor cells. In fact this experiment is underway.

One of the major emphasis in this project is that MHC class II^+ transfectants will be able to activate tumor-specific $CD4^+$ T cells which might provide help to the $CD8^+$ effector T cells. Towards this goal, 66.1/I-A^{d+} transfectants are being generated. Once the stable transfectants are available they will be cloned and used in the in vivo tumor challenge and protection experiments. In addition, it is important to investigate whether transfectants having both B7-1 (or B7-2) and MHC class II molecules are better immunogens than those with either of the molecules alone.

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Figure Legends

Figure 1

Balb/c mice recieved indicated number of tumor cells s.c. on day 0. Each group contained 5 mice. Tumor growth was measured every 5-8 days following tumor challenge by mesuring the perpendicular diameters with a caliper. Tumor volume was calculated using the formula πr^3, where r was computed by dividing the sum of the perpendicular diameters with 4.

Figure 2

Triplicate cultures of 66.1/WT tumor cells were set up in 96-well plates in complete medium in the presence of different concentrations of either G418 or Hygromycin. Two different concentrations of cells were tested. Proliferation of cells was measured by 3H-thymidine uptake during the last 18 hr of the 3 day culture period.

Figure 3 A

Bulk cultures of 66.1/B7.I transfectants were stained with either mouse anti-rat-FITC conjugate alone (panels 1 and 3) or stained with a rat mAb (1G10) against murine B7.I and then with the mouse anti-rat FITC conjugate (panels 2 and 4). Similarly, 66.1/B7.I transfectants were stained with either goat anti-mouse-FITC conjugate alone (panels 5 and 7) or stained with a mAb against murine Kd molecule (31.3.4s) and then with goat anti-mouse-FITC conjugate (panels 6 and 8). The x-axis represent 3 log fluorescence and the y-axis represent relative number of cells.

Figure 3 B

Three separately derived 66.1/B7.I$^+$ lines were tested for stable expression of B7.I molecule, as described in Figure 3A.

Figure 4

Balb/c mice received s.c. indicated numbers of live B7.I$^+$ transfected tumor cells on day 0. Three different lines of transfectants were injected and the tumor growth was monitored as described in Fig. 1.

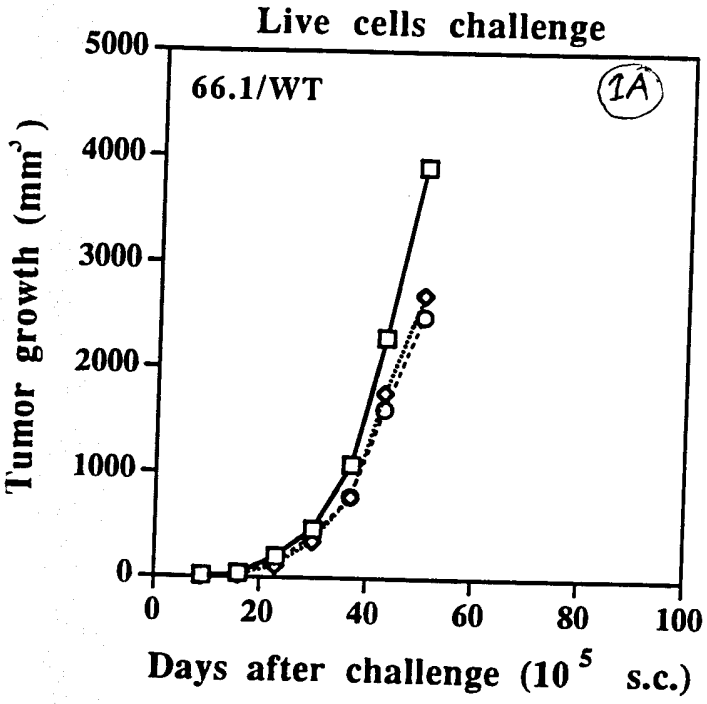
Figure 5

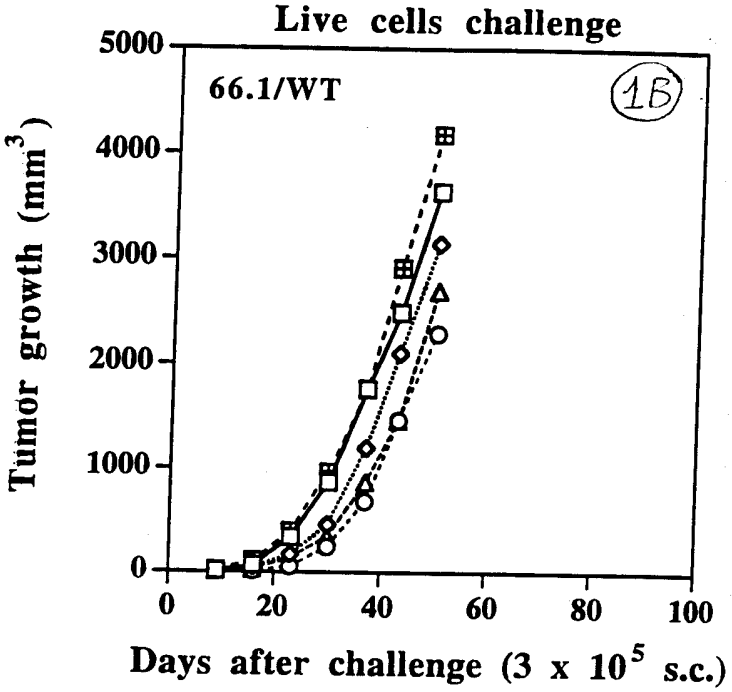
66.1/WT tumor cells were subjected to various doses of radiation and were cultured at indicated cell concentrations in 96-well plates. Proliferation was assessed as described in Fig. 2.

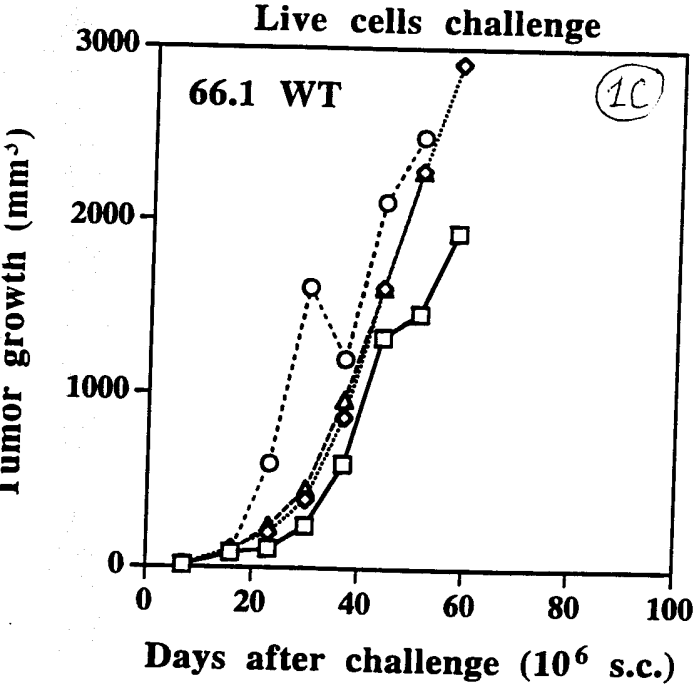
Figure 6

Balb/c mice were either unprimed or primed s.c. with 3-5 x 10^5 66.1/WT tumor cells that were growth arrested by a predetermined dose of irradiation, 30,000 rad. No tumor growth was seen and 80 days later both groups were challenged s.c. with 3 x 10^5 66.1/WT tumor cells. The tumor growth following challenge was monitored as in Fig. 1.

Figure 1


Live cells challenge
66.1/WT
1A
Tumor growth (mm³)
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
Days after challenge (10⁵ s.c.)


Live cells challenge
66.1/WT
1B
Tumor growth (mm³)
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
Days after challenge (3 x 10⁵ s.c.)


Live cells challenge
66.1 WT
1C
Tumor growth (mm³)
3000
2000
1000
0
0
20
40
60
80
100
Days after challenge (10⁶ s.c.)

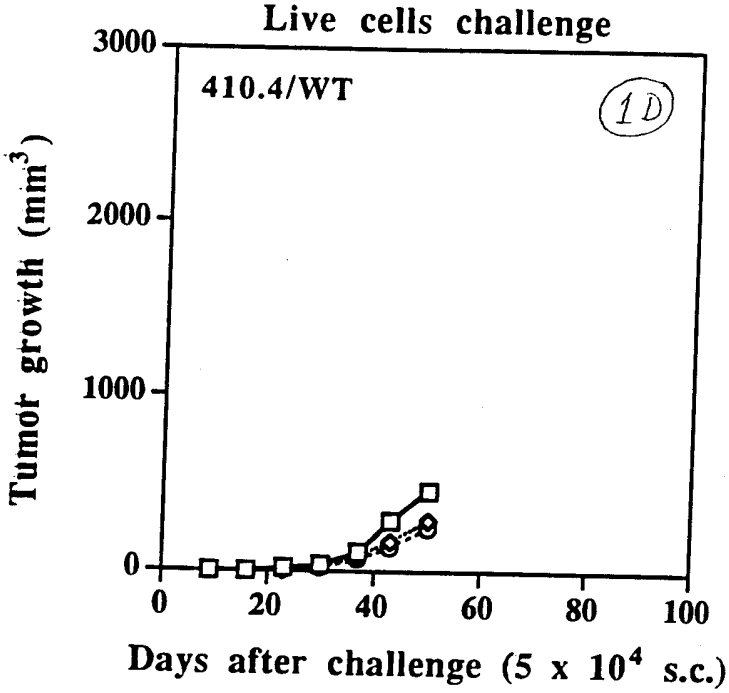
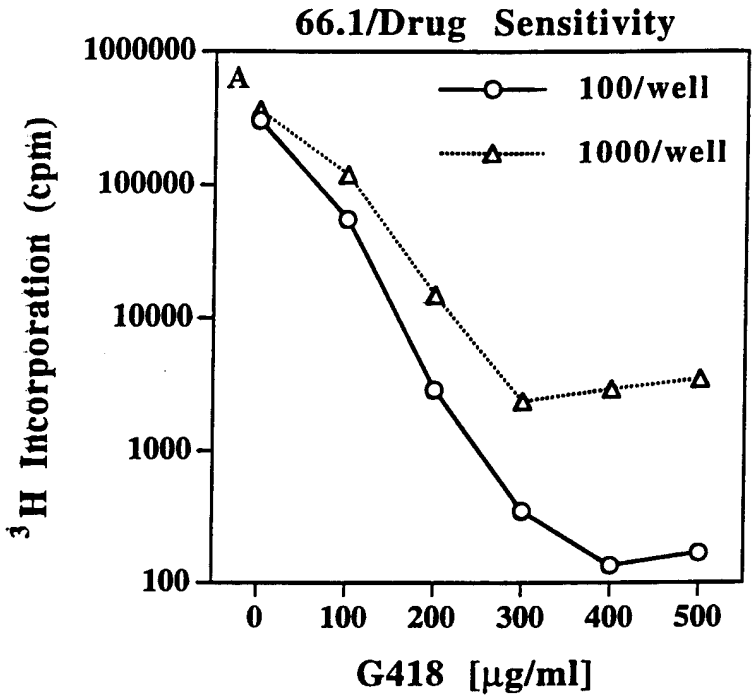

Live cells challenge
410.4/WT
1D
Tumor growth (mm³)
3000
2000
1000
0
0
20
40
60
80
100
Days after challenge (5 x 10⁴ s.c.)

Figure 2


66.1/Drug Sensitivity
A
100/well
1000/well
^{3}H Incorporation (cpm)
1000000
100000
10000
1000
100
0
100
200
300
400
500
G418 [µg/ml]

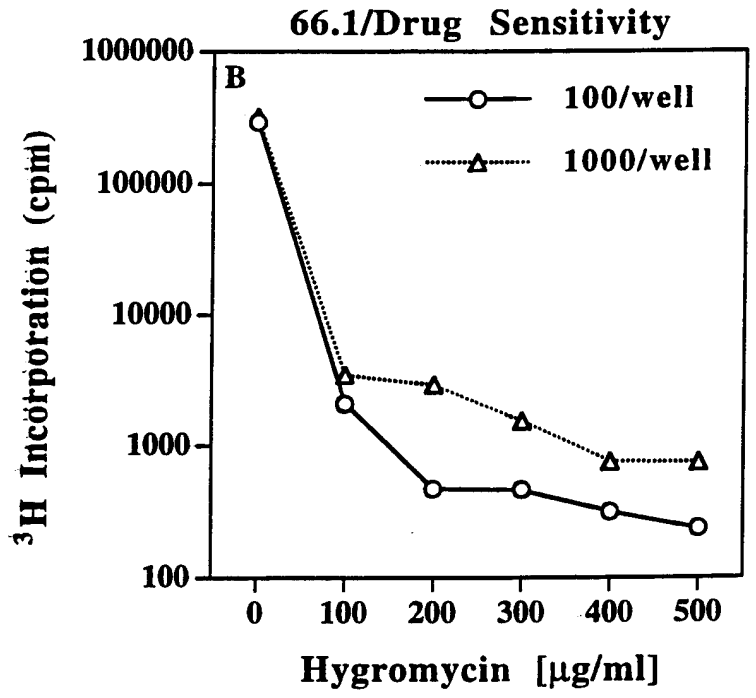

66.1/Drug Sensitivity
B
100/well
1000/well
^{3}H Incorporation (cpm)
1000000
100000
10000
1000
100
0
100
200
300
400
500
Hygromycin [µg/ml]

Figure 3 A

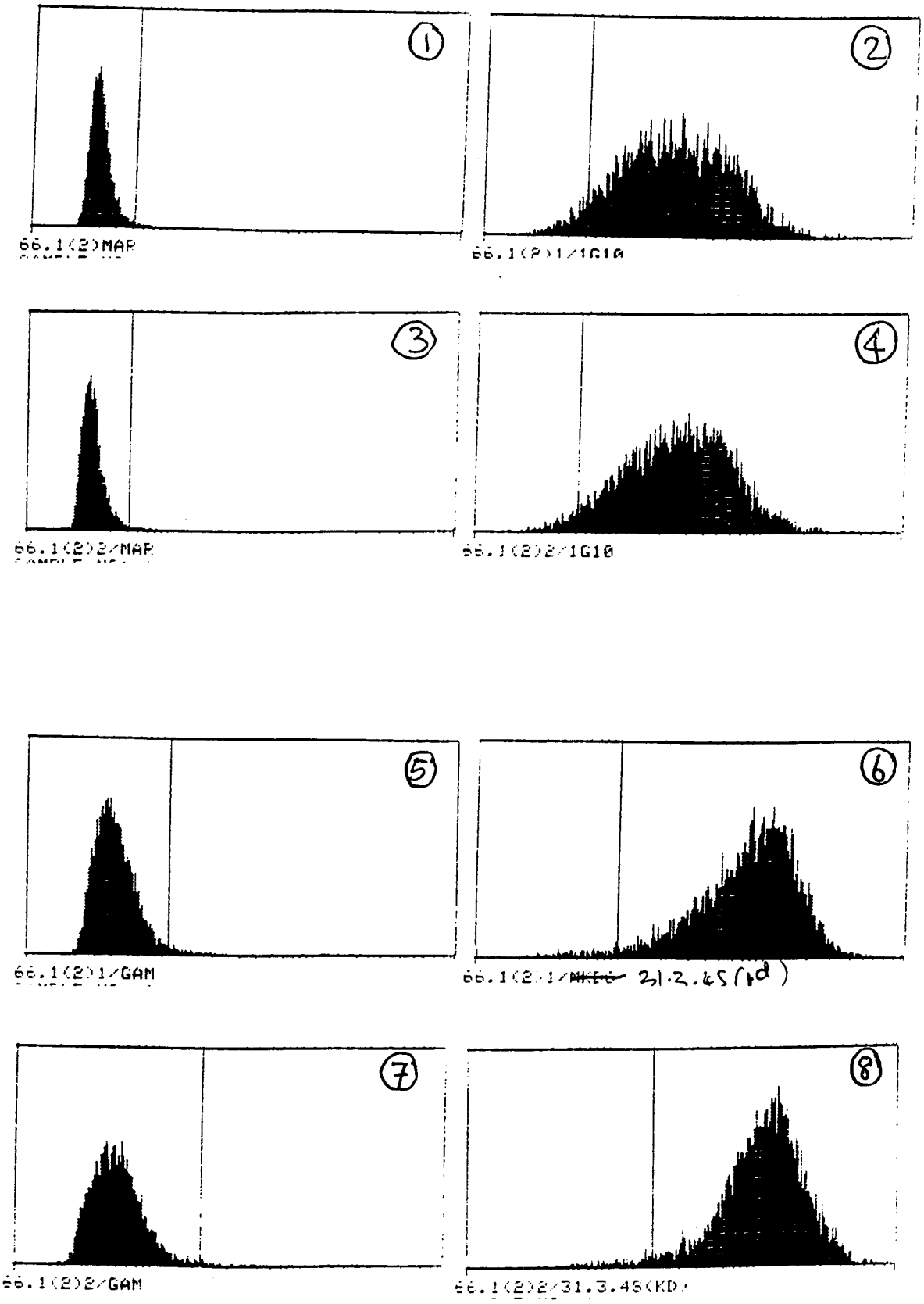

①
②
66.1(2)MAP
66.1(2)1/1G10
③
④
66.1(2)2/MAP
66.1(2)2/1G10
⑤
⑥
66.1(2)1/GAM
66.1(2)1/~~MIEC~~ 31.2.65(Rd)
⑦
⑧
66.1(2)2/GAM
66.1(2)2/31.3.48(KD)

Figure 3 B

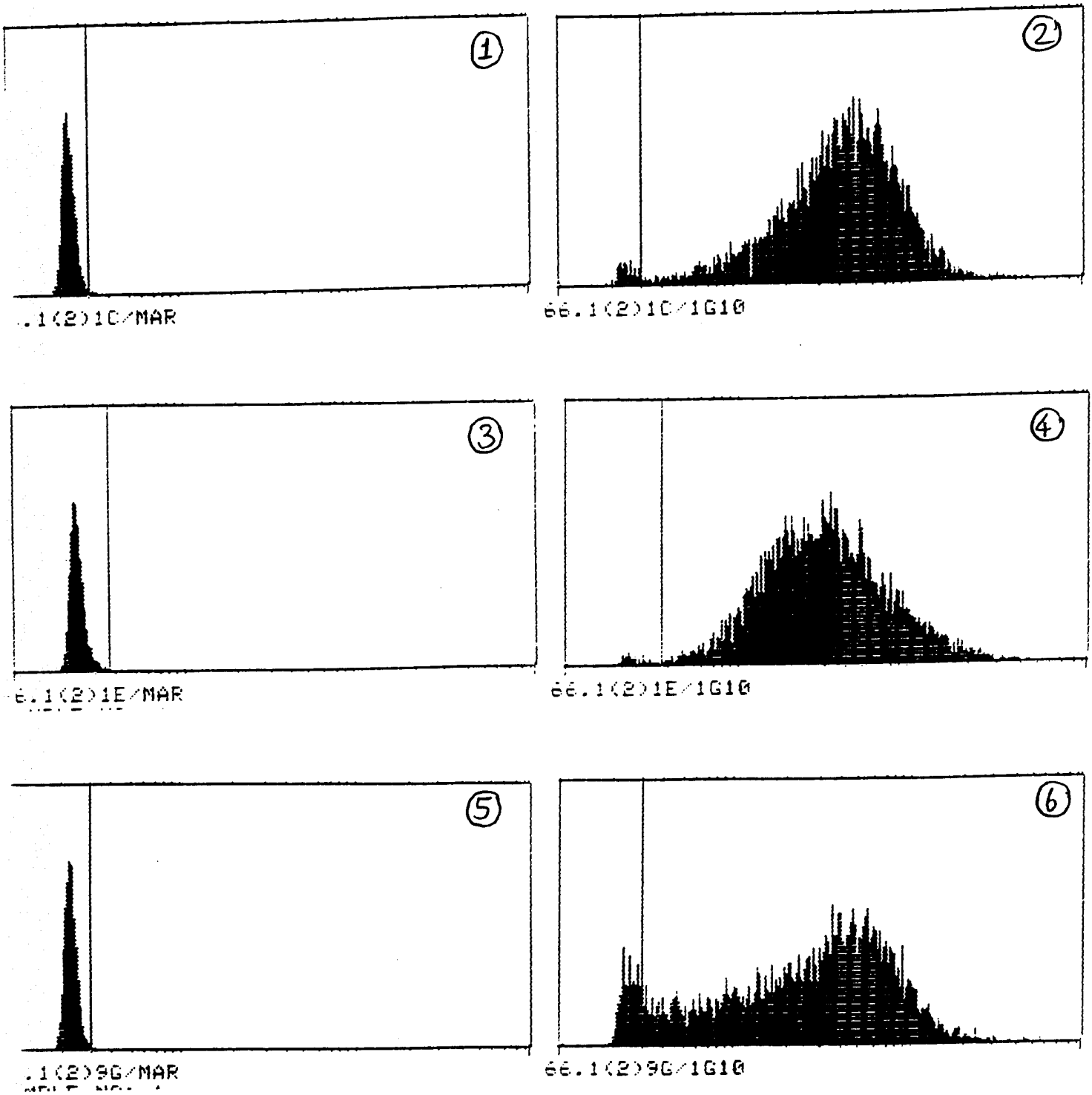
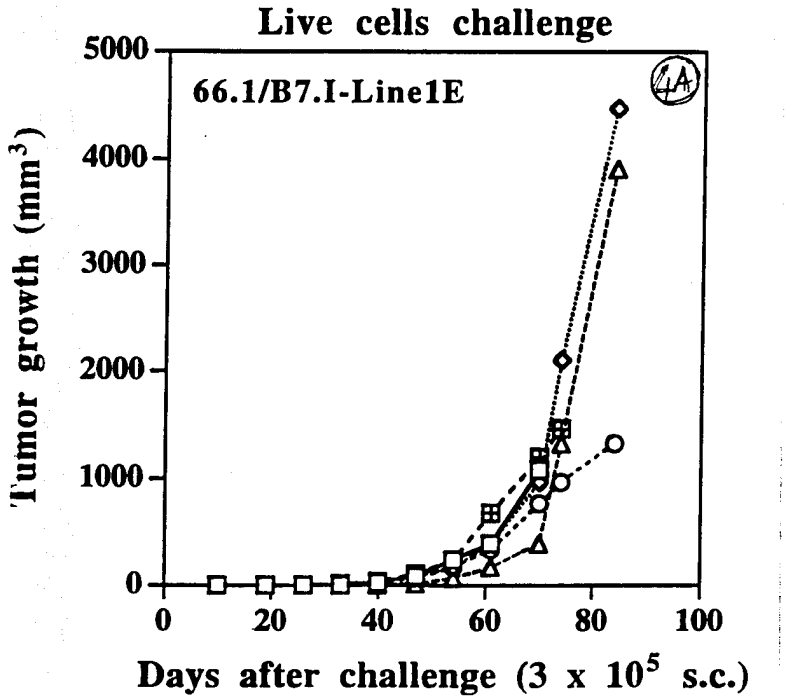
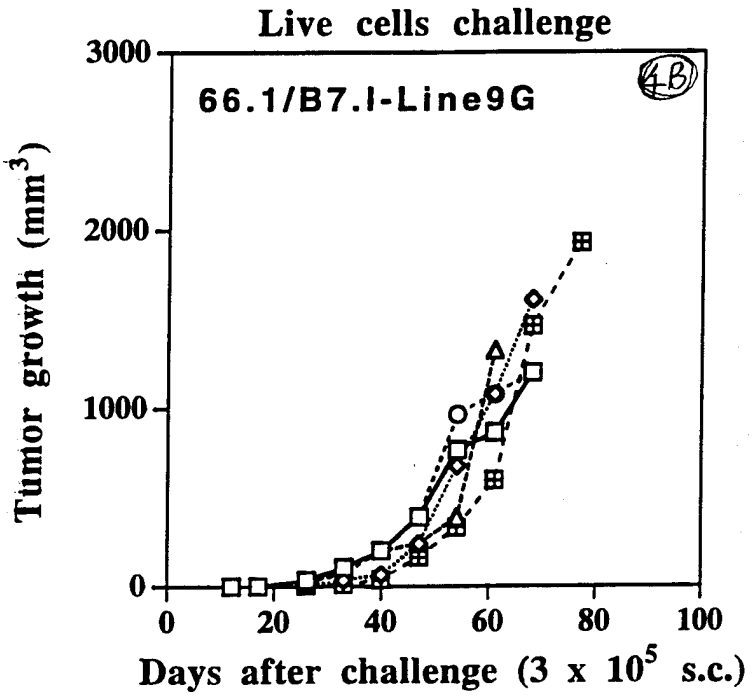
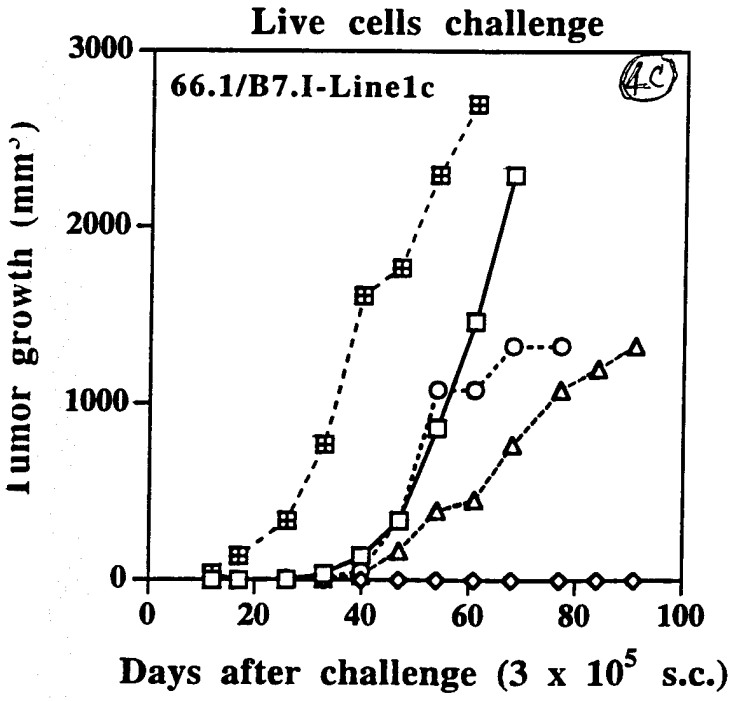

①
.1(2)1C/MAR
②
66.1(2)1C/1G10
③
6.1(2)1E/MAR
④
66.1(2)1E/1G10
⑤
.1(2)9G/MAR
⑥
66.1(2)9G/1G10

Figure 4


Live cells challenge
66.1/B7.I-Line1E
4A
Tumor growth (mm3)
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
Days after challenge (3 x 10^5 s.c.)


Live cells challenge
66.1/B7.I-Line9G
4B
Tumor growth (mm3)
3000
2000
1000
0
0
20
40
60
80
100
Days after challenge (3 x 10^5 s.c.)


Live cells challenge
66.1/B7.I-Line1c
4C
Tumor growth (mm3)
3000
2000
1000
0
0
20
40
60
80
100
Days after challenge (3 x 10^5 s.c.)

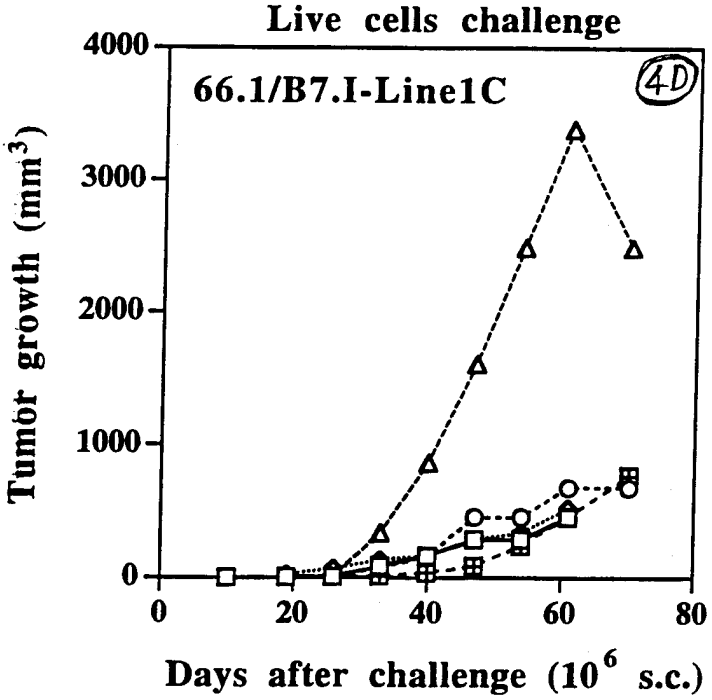

Live cells challenge
66.1/B7.I-Line1C
4D
Tumor growth (mm3)
4000
3000
2000
1000
0
0
20
40
60
80
Days after challenge (10^6 s.c.)

Figure 5

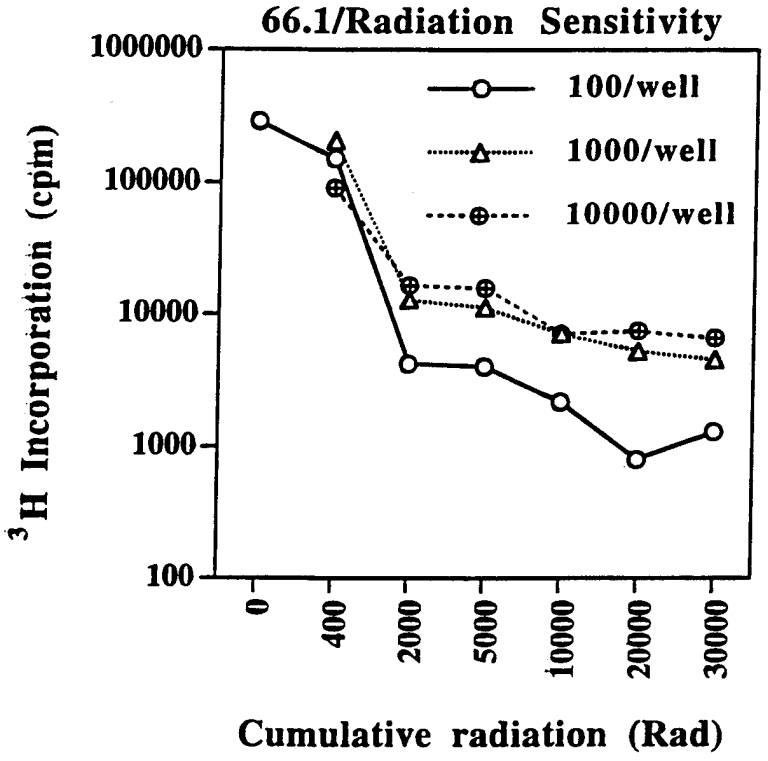
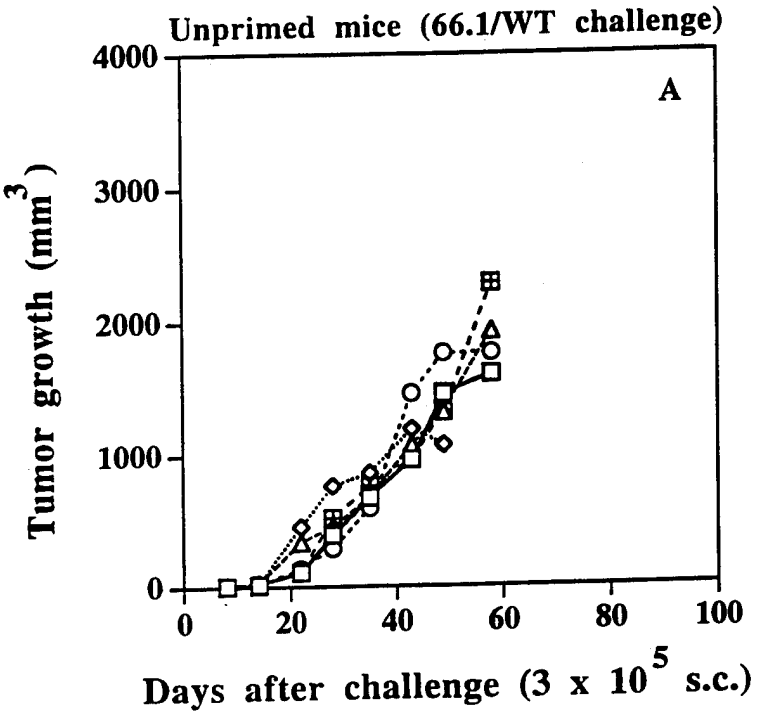
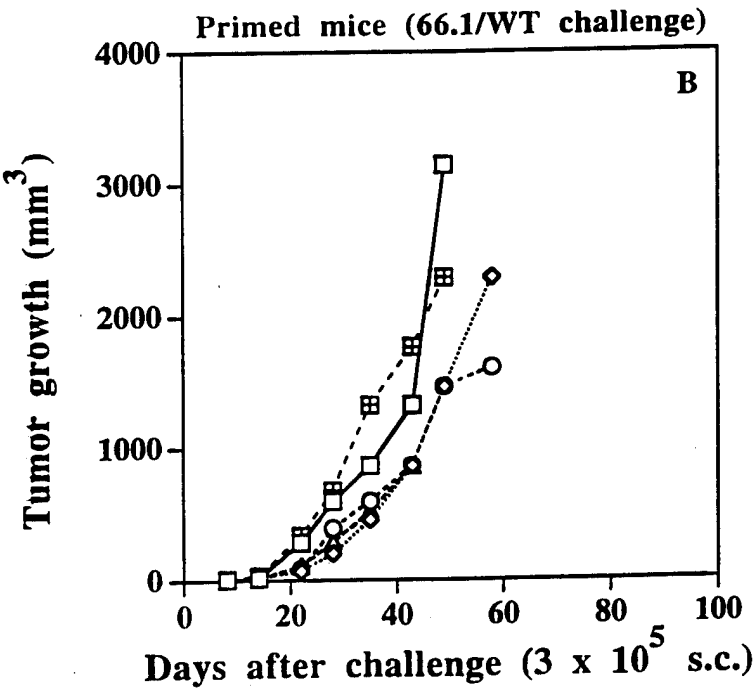

66.1/Radiation Sensitivity
1000000
100000
10000
1000
100
^{3}H Incorporation (cpm)
100/well
1000/well
10000/well
0
400
2000
5000
10000
20000
30000
Cumulative radiation (Rad)

Figure 6


Unprimed mice (66.1/WT challenge)
A
Tumor growth (mm3)
4000
3000
2000
1000
0
0
20
40
60
80
100
Days after challenge (3 x 10^5 s.c.)


Primed mice (66.1/WT challenge)
B
Tumor growth (mm3)
4000
3000
2000
1000
0
0
20
40
60
80
100
Days after challenge (3 x 10^5 s.c.)