Cancer Immunol Immunother (1991) 33: 267–273
0340700491000627

Cancer
Immunology
Immunotherapy

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# Immunogenicity of a non-class I MHC expressing murine tumor transfected with the influenza virus hemagglutinin or murine interleukin-2 genes*

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Received 8 November 1990/Accepted 15 February 1991

**Summary.** The transfection of murine SP1 tumor cells with the hemagglutinin (*HA*) gene of influenza virus results, after fluorescent-activated cell sorting (FACS), in the selection of high-HA-expressing cell lines called H4A and H4B. Both lines fail to grow in syngeneic animals at doses that result in 100% tumor take of non-transfected tumor cells. Both grow in immunosuppressed mice. SP1 and H4A or H4B cells express few class I major histocompatibility complex (MHC) antigens but do express class II $IA^k$ antigens. H4A or H4B cells engender a cytotoxic T lymphocyte (CTL) response but cannot protect against a challenge with SP1 cells. This CTL response is inhibited by anti-CD4 but not anti-CD8 antibodies. Using FACS, we were able to select a population (called H5AK5) with high class-I MHC antigen expression. Like H4A and H4B, H5AK5 cells fail to grow in syngeneic animals but do grow in immunosuppressed mice. However, unlike H4A or H4B, H5AK5 can induce protection against a challenge with $1 \times 10^5$ SP1 cells. These studies indicate that the immunogenicity of *HA*-transfected SP1 cells may correlate with the cell-surface expression of class II MHC antigens. However, HA-expressing SP1 cells seem able to induce a protective response against a parent SP1 cell challenge only if they also express class I MHC antigens. This view is supported by the observations that SP1 cells expressing murine interleukin-2 do not express class I MHC antigens, fail to grow in syngeneic animals, do grow in immunosuppressed mice but do not protect against a challenge with parental SP1 cells.

**Key words:** Major histocompatibility complex – Transfected genes – Immunogenicity – Hemagglutinin

## Introduction

We previously showed that transfection of the murine colon tumor CT-26 with the gene (*HA*) coding for the influenza HA antigen produces highly immunogenic variants [4]. *HA*-transfected cells fail to grow in syngeneic hosts, grow in nude mice, and protect against a challenge with parent non-transfected CT-26 cells. Protection against the parent cell is systemic [16], in that CT-26 fail to grow in immunized animals if injected intraportally, i.v., s.c. or intracecally. In additiion, transfected cells retain their immunogenicity after X-irradiation [6].

Because our goal is to determine whether this approach could be applicable to human disease, several additional difficulties have to be overcome. One problem involves the need for cloning *HA*-transfected cells [4, 16]. A second issue relates to whether the immunogenicity of transfected cells relies on the surface expression of class I major histocompatibility complex (MHC) antigens, since many human tumors are thought to express little or no surface class I MHC antigens (reviewed in [20]). In addition, human tumors are believed to be poorly immunogenic and thus not to express tumor antigens of sufficient quantity or quality to induce an immune response.

Since our initial transfection studies involved the CT-26 tumor, a chemically induced weakly immunogenic murine cell line that expresses high levels of class I MHC antigens, we expanded our analyses to include a spontaneous murine tumor. The CBA-SP1 mammary adenocarcinoma is poorly (if at all) immunogenic. In addition, only 7%–12% of SP1 cells express surface class I MHC antigens [1] (and this manuscript). In this report, we show that the SP1 tumor can be made immunogenic after transfection with *HA* and that HA-expressing cells can be used as immunogens for pro-

* This work was supported by The Clayton Fund, The Sid W. Richardson Foundation and PHS grants CA 39853 and 41525. Toshiyuki Itaya is a visiting scientist supported by the Smith Education Fund of the Department of Cell Biology. Troy Fiesinger is a summer research investigator sponsored by The University of Texas M. D. Anderson Cancer Center Summer Program for College Students

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tection against a challenge with $1 \times 10^4$ parental (non-transfected) cells. This protective ability can be increased 50-fold if the immunizing cells express class I MHC antigens as well as HA.

The view that class I MHC antigens are needed to provide a protective response is further supported by experiments in which SP1 cells secreting interleukin-2 (SP1/IL-2) were found to be non-tumorigenic, though they expressed little if any class I MHC antigens. SP1/IL-2 could not, however, protect against a challenge with parental SP1 cells.

## Materials and methods

*Mice.* CBA female mice between 6 and 8 weeks of age were obtained from the Frederick Animal Facility of the National Cancer Institute.

*Tumor.* The origin of the CBA-SP1 tumor has been described previously [1]. SP1 arose as a spontaneous mammary ductal carcinoma in an 18-month-old ex-breeder CBA mouse. SP1 grows readily after s. c. or i. v. inoculation and is poorly if at all immunogenic.

*Culture conditions.* Cells were cultured in Gibco RPMI-1640 medium supplemented with 50000 U penicillin and streptomycin, 150 mg L-glutamine, 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 375 mg sodium bicarbonate, and 7% fetal calf serum (Hazelton Laboratories, Denver, Colo.). The allogeneic B16 melanoma of C57BL/6 mice and MDW1 tumor of DBA/2 mice were used as controls and were maintained in the same medium as that used for the SP1 cells. All tumors were tested periodically for the presence of *Mycoplasma* using the Gen Probe RNA hybridization method (Gen Probe, San Diego, Calif.); contamination with *Mycoplasma* was never observed. In addition, the cells were tested with a murine antibody production test (Microbiological Associates, Bethesda, Md.) and found to be free of 13 murine pathogenic viruses.

*Antisera.* The monoclonal antibodies used in these studies were as follows. Murine anti-$D^k/K^d$ (clone 15-5-5S) was kindly provided by Dr. B. E. Elliott (Queen's University, Kingston, Ontario, Canada). Murine anti-$K^k$ (clone 16-1-11N) was purchased from the Litton Company (Charleston, S. C.). Murine anti-$K^k/D^k$ Ab (clone H 100-27/55), anti-I-$A^k$ (clone 14V18), and anti-I-$E^k$ (clone 14-4-4S) were purchased from the Cedarlane Company (Ontario, Canada). High-titer rabbit anti-H2N2 influenza virus serum was generously provided by Dr. M. J. Gething (The University of Texas at Dallas). This antiserum is specific for the A/Jap/305/57 H2N2 influenza virus HA and was used at a 1:300 dilution. Anti-CD8 and anti-CD4 antibodies were obtained from Becton-Dickinson (Mountainview, Calif.). The anti-CD8 was an anti-Lyt2 from hybridoma 53-6.7. The anti-CD4 was anti-(mouse L3T4) from hybridoma clone GK1.5. These antisera were screened for effectiveness against normal lymph node and spleen cells prior to use.

*Transfection of cells with DNA.* DNA was introduced into SP1 cells as a coprecipitate with calcium phosphate [4, 5]. The SP1-Neo cell line was obtained by transfection of a mixture of 1 μg plasmid vector pSV2neo [12] with 10 μg CBA mouse liver DNA as carrier. The SP1-HA cell lines were each obtained by transfection with a mixture of 1 μg pSV2neo, 10 μg pBV-1MTHA [4], and 10 μg CBA liver DNA. pBV-1MTHA is a bovine papillomavirus-expression vector containing the metallothionein promoter and *HA* gene and has been described previously [15]. The BPV-SP1 cell lines were obtained by transfection with a mixture of 1 μg pSV2neo and 10 μg pBPV-BV1, a bovine papillomavirus-expression vector *without* the metallothionein promoter or the *HA* gene. After exposure to the precipitate for 4 h, cells were washed once with HEPES-buffered saline (HBS) and then shocked for 3 min at room temperature with a 25% solution of dimethylsulfoxide in HBS. The cells were then washed twice with HBS and incubated 48 h in RPMI-1640 medium with 10% fetal bovine serum at 37°C. Selection in G418 (0.5 mg/ml) was then begun and surviving colonies were visible after approximately 14 days.

The SP1/IL-2 cell line was obtained by transfection with 10 μg plasmid vector pBCMG-neo-mIL-2, a BPV expression vector containing a murine IL-2 cDNA clone under the transcriptional control of a cytomegalovirus promoter with a rabbit β-globin introns, splice and poly(A) addition signals. The plasmid also contains the *TnS* neomycin-resistance gene [9, 10]. Control cells were transfected with the pCMV-neo-BAM vector from which the IL-2 cDNA had been removed.

*Flow cytofluorometry.* Quantitative analysis of HA or H-$2^k$ expression on cell surfaces and preparative sorting of cells on the basis of HA expression were performed using a FACS [4]. Cells were scraped from tissue-culture plates using a rubber policeman and incubated with rabbit anti-influenza virus antibody or an anti-H-$2K^k$ antibody for 30 min at 4°C. After repeated washings, the cells were incubated with FITC-conjugated goat anti-(rabbit IgG) or goat anti-(mouse IgG) antibodies at 4°C for 30 min. After further washing, the cells were examined using cytofluorometry.

*IL-2 assays.* Supernatants of transfected cells were assayed for IL-2 by the method described by Janis [8]. Dilutions of supernatants were transferred to 96-well microtiter plates containing 3000 CTLL-2 cells/well. After 24 h [$^3$H]thymidine was added for 12 h and its incorporation assessed using a cell harvester. IL-2 whas assayed as U/ml, calculated as the reciprocal of the supernatant dilution giving half-maximal proliferation of CTLL-2.

*H4A and H4B cell lines.* HA-transfected CBA-SP1 cells were incubated with rabbit anti-HA antisera and FITC-conjugated goat anti-(rabbit IgG) and sorted by FACS. The most fluorescent 5% of the cells were collected and expanded in vitro. The procedure was repeated four times. The cells obtained after four sorts were expanded in culture and designated H4A and H4B. H4A and H4B were obtained from different transfection plates (pooled colonies). These sublines were maintained in the same medium as that used for SP1 cells except for the addition of G418 (0.5 mg/ml). H4AK3, H4AK4, and H5AK5 were obtained from the 3%–7% $K^k$-antigen-positive population derived from the H4A cell line. The most fluorescent 2% of the $K^k$-positive cells were cell-sorted three (H4AK3) or four (H4AK4) times. The H5AK5 cell line was obtained from double-sorted (H2 and HA) H4AK4 cells after double selection of the most fluorescent 5% of both HA- and $K^k$-expressing cells.

*In vitro interferon γ treatment.* To augment native H-2 expression of SP1 or H4A cells, the cells were treated with mouse interferon γ (IFNγ). Recombinant murine IFNγ was obtained from Genentech Inc. (San Francisco, Calif.). Samples of $3 \times 10^5$ tumor cells were divided among 10-cm plastic dishes and incubated with 100 U/ml IFNγ for 4 days. On the 4th day, IFNγ-treated cells were divided and reincubated with 100 U/ml IFNγ for an additional 3 days. These IFNγ-treated cells were analyzed for the expression of class I or class II H-2 antigens and were injected into normal mice to assess their immunogenicity. SP1 parent and HA-expressing cells were all resistant to the cytotoxic effects of IFNγ and grew normally in medium containing 100 U/ml IFNγ.

*Assessment of T cell cytotoxicity.* T cell cytotoxicity was measured using a previously described [$^{111}$In] indium-oxine-release assay [21].

*Transplantation studies.* To evaluate the immunogenic potential of *HA*-transfected cells, $1 \times 10^4 - 5 \times 10^5$ viable HA-positive cells were injected into the left flank of CBA mice. The challenge dose of CBA-SP1 cells was injected s.c. 10–14 days later in the right flank, and the presence or absence of tumor growth was assessed. In addition, H4A or H4B cells that had been irradiated with 12000 R were used to immunize CBA mice against an SP1 challenge. To assess the role of the immune response in the rejection of HA-expressing cells, we injected these cells into CBA mice that had been X-irradiated with 600 R. The number of tumor cells required for lethal tumor growth in 50% of the animals was estimated by the method of Reed and Muench [14] and expressed as the $LD_{50}$. The statistical difference in the survival rate between groups was calculated by the $\chi^2$ test.

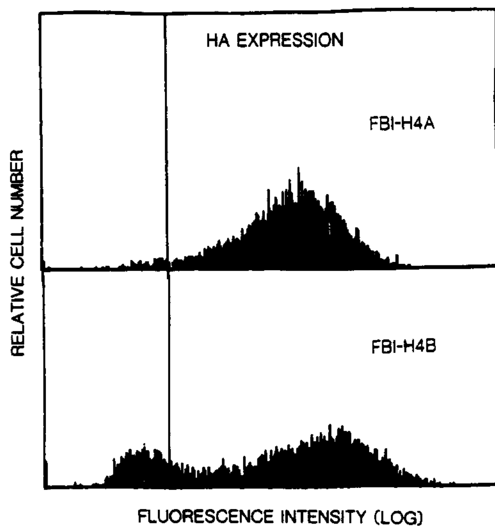


**Fig. 1.** Cell-surface expression of hemagglutinin (HA) as shown by fluorescence intensity distribution based on the binding of fluorescein-labeled goat anti-(rabbit immunoglobulin) to cells pre-treated with anti-HA antibody

## Results

H4A and H4B cells were obtained using fluorescence-activated cell sorting (FACS) selection (four times) of the most brightly stained 5% of cells reactive with anti-HA antibody. Figure 1 demonstrates that for H4A, the expression curve has a single peak, with 92% of H4A cells expressing HA. In contrast, H4B has a biphasic HA-expression curve, with 75% of the cells expressing HA despite selection of the brightest HA-expressing cells from the extreme right of the curve on four occasions. These cells inevitably reverted to a biphasic expression curve. Neither $ZnCl_2$ (the HA gene is linked to a metallothionein promoter in the pBPV-HA vector) [4] nor IFN-γ could increase HA expression (data not shown).

Table 1 shows that H4A and H4B cells are immunogenic and that their immunogenicity is related to the dose of H4A or H4B cells injected. The injection of $1 \times 10^4$ cells results in essentially no tumor growth; $5 \times 10^4$ cells produce tumor growth in 64% of mice injected with H4B and 33% of those injected with H4A; and injection of $1 \times 10^5$ cells produces tumor growth in almost all animals despite HA expression.

It had previously been reported that the SP1 tumor expresses little or no surface class I MHC [1]. In addition, Elliot et al. [1, 3] had reported that the immunogenicity of SP1 could be shown to correlate with increased H-2 expression. We therefore determined whether murine IFNγ, a known MHC inducer, could augment the immunogenicity of SP1 cells by increasing either H-2 or HA expression. (We note here that SP1 cells transfected with pSV2neo alone did not express increased H2 and were not immunogenic) [12]. SP1 as well as H4A and H4B cells were treated with IFNγ, followed by FACS analysis. In no case was IFNγ able to increase HA expression (data not shown). Figure 2 a, b illustrates that IFNγ was able to increase $H2K^k$ and $HsD^k$ expression by the parent SP1 cells dramatically. H4A cells showed some increase in class I H-2 expression after IFNγ treatment (Fig. 2 a, b), but H4B cells demonstrated no change in class I H-2 expression (Fig. 2 a, b). The reasons for this diminished reponse to IFNγ of the H4A and H4B cell lines are currently unknown. Fig. 2 c, d demonstrates that SP1 cells express class II MHC antigens but only $IA^k$ and not $IE^k$. The expression of class II MHC antigens was not appreciably affected by IFNγ.

Despite evidence for little class I MHC expression, both H4A and H4B cells could engender a cytotoxic T lymphocyte (CTL) response. Data for the H4B response is shown in Table 2. This table also shows that the addition of anti-CD4, but not anti-CD8 antibodies, to the effector lymphocytes prior to their addition to the SP1 targets, reduced the percentage cytotoxicity by approximately 50% (we emphasize the the SP1 targets were analyzed by FACS on the day of the assay and expressed little if any $H2^k$, but all

**Table 1.** Immunogenicity of *HA*-transfected SP1 cells in syngeneic CBA mice[a]

| Cell lines | No. of mice with tumor/no. of mice injected | | | | $10^{-3} \times LD_{50}$[b] |
|---|---|---|---|---|---|
| | Challenge dose | | | Immunosuppressed nude mice | |
| | $1 \times 10^4$ cells | $5 \times 10^4$ cells | $1 \times 10^5$ cells | $1 \times 10^4$/cells | |
| SP1-HA | 4/ 5 | 5/ 5 | 5/ 5 | | 4.2 |
| H4A | 1/16 | 4/12[c] | 9/12 | 8/8 | 64 |
| H4B | 0/16 | 9/14 | 12/12 | 6/6 | 35 |
| BPV-SP1[d] | 5/ 5 | 5/ 5 | 5/ 5 | | 3.2 |
| pSV2neo[e] | 8/10 | 5/ 5 | 5/ 5 | | 4.2 |
| SP1 | 15/15 | 10/10 | 10/10 | | 10.1 |

[a] Groups of CBA mice were injected s.c. with different doses of *HA*-transfected and control cells. Tumor growth was assessed by observation
[b] The number of tumor cells required for lethal growth in 50% of syngeneic mice
[c] $P < 0.001$, compared with parent SP1 tumor
[d] Co-transfected with BPV-BV1 and pSV2neo. BPV-BV1 does not contain the MTHA sequence
[e] Transfected with pSV2neo alone

SP1-HA = transfected SP1 cells prior to FACS purification

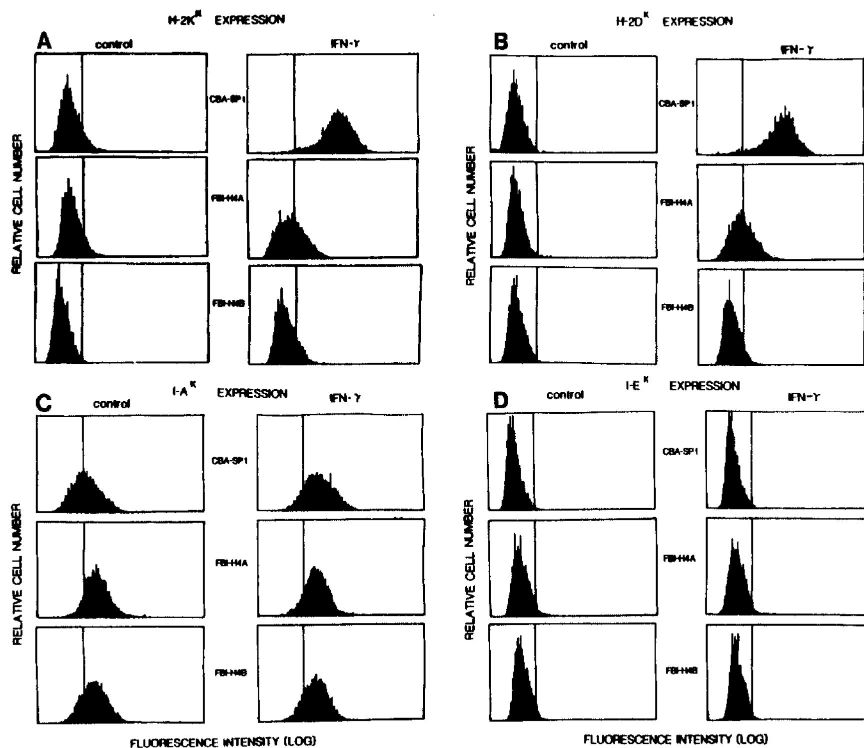


**Fig. 2.** Cell-surface expression of class I and class II major histocompatibility complex (MHC) antigens before and after interferon-γ treatment. Cells were treated with murine anti-H2K$^k$, D$^k$, I-A$^k$ or I-E$^k$, washed and treated with fluorescein-labeled goat anti-(mouse IgG). Cell-surface expression of MHC antigens is shown by fluorescence intensity distribution

target cells expressed I-A$^k$). These data suggest (but do not conclusively prove) that class II H2 restriction may play a role in the response to these HA-expressing cells.

*Protective effect of H4A and H4B against a challenge with SP1*

We reasoned that since the H4A and H4B lines were immunogenic, they might be able to protect against a challenge with parent SP1 cells. The results of these studies are shown in Table 3 and can be summarized as follows. Neither H4A nor H4B cells could protect against a challenge with SP1 cells. However, because H4A and H4B fail to grow only when injected in doses smaller than $5\times10^4$ cells (Table 1), only low immunizing doses of viable cells could be used. We therefore X-irradiated H4A and H4B, and immunized animals with $(3-5)\times10^6$ cells given s.c. one to three times. This resulted in a statistically significant increase in protection against an SP1 challenge, but the effect was of questionable biological relevance. No effect was seen after immunization with X-irradiated parental SP1 cells or an allogeneic tumor.

In Fig. 2a we show that both the parent SP1 and HA-expressing H4A lines contained a small number (7%–12%) of cells that express H-2K$^k$. We obtained a population of cells expressing H-2K$^k$ and HA, by multiple FACS selections of H4A cells using an anti-H-2K$^k$ antibody and obtained the H5AK5 cell line after five such selections. Sixty-six percent of the H5AK5 cells express H-2K$^k$ and 84% express HA at a level comparable to that seen with H4A cells (data not shown).

**Table 2.** Cell-mediated cytotoxicity engendered after H4B immunization[a]

| In vivo primary immunization | In vitro secondary stimulation | Target | Antibody | Cytotoxicity (%) | | |
|---|---|---|---|---|---|---|
| | | | | 10:1 | 50:1 | 100:1 |
| X-irradiated H4B | H4B | H4B | – | 11.3 | 23.8 | 31.9 |
| | | SP1 | – | 9.7 | 34.3 | 29.5 |
| | | SP1 | Anti-CD8 | 13.1 | 26.5 | 22.8 |
| | | SP1 | Anti-CD4 | 7.1 | 17.0 | 12.7 |
| | | MDW1 | | 0 | 0 | 2 |
| Viable MDW1 | MDW1 | MDW1 | | 60 | 100 | 100 |

[a] Groups of CBA mice were immunized with X-irradiated HA-expressing H4B cells. Spleen cells were removed 14 days later and restimulated in vitro with mitomycin-C-treated H4B cells. After 5 days, the spleen cells were harvested and mixed with $^{111}$In-labeled targets at a 10:1, 50:1, or 100:1 lymphocyte-to-tumor cell ratios. In some experiments, the effector lymphocytes were incubated for 30 min with anti-CD4 or anti-CD8 antibody prior to their addition to the radiolabeled targets. MDW1 is a known immunogenic variant of a DBA/2 tumor that was used as a control

**Table 3.** Immunoprotection by H4A or H4B immunization against a challenge with parent SP1 cells[a]

| Immunizing cells | Immunization dose | No. of immunizations | SP1 challenge: no. of mice with tumor/ no. of mice injected |
|---|---|---|---|
| Viable cells | | | |
| H4A | $1\times10^4$ | 2 | 8/ 9 |
| H4B | $1\times10^4$ | 2 | 8/ 9 |
| X-irradiated cells | | | |
| H4A | $3\times10^6$ | 2 | 8/15 |
| H4A | $3\times10^6$ | 3 | 10/15 |
| H4B | $5\times10^6$ | 2 | 6/ 8 |
| SP1 | $5\times10^6$ | 3 | 8/ 9 |
| Allogeneic B16 melanoma | $5\times10^6$ | 1 | 6/ 6 |
| No immunization | | | 15/15 |

[a] Groups of CBA mice were immunized with viable or X-irradiated (12 000 R) *HA*-transfected or parent cells. After 2 weeks, all groups were challenged s.c. with $1\times10^4$ SP1 cells and observed for tumor growth. Samples of $1\times10^4$ SP1 cells were injected s.c. 21 days after a single immunization or 7 days after the last of two or three immunizations. $P$ = <0.01 (H4A, $3\times10^6$ twice) and <0.05 (H4A, $3\times10^6$, three times) using the $\chi^2$ test

Table 4 shows that the H5AK5 line is as immunogenic as H4A when $1\times10^4$ cells are injected. At higher doses, H5AK5 is more immunogenic than H4A. The calculated $LD_{50}$ is almost 4.5 times greater for H5AK5 than for H4A. H5AK5 cells do grow in immunosuppressed mice. Normal CBA mice immunized with X-irradiated H5AK5 cells on three occasions ($3\times10^6$ cells) were able to protect 50% of the animals (7 of 14) against a challenge with $1\times10^4$ parent SP1 cells. Because of this relatively low protective effect and because of the need to use X-irradiated H5AK5 cells (viable H5AK5 cells invariably grow at doses of $3\times10^6$ cells) we decided to clone the H5AK5 cells.

We examined 19 independent clones (Table 5, next page) that could be divided into three groups. Group I clones were highly immunogenic and protected against a challenge with SP1 cells. Group II clones were immunogenic but could not protect against an SP1 challenge. Group III clones were tumorigenic.

We next attempted to correlate HA and/or H-2K$^k$ expression with the immune phenotype of all the clones. Several general conclusions can be drawn from these studies. In group I, three of three clones were high expressers of HA. In group III, all the clones were low expressers of both HA and H-2K$^k$. Group II clones were immunogenic but did not protect against an SP1 challenge. This group also exhibited generally lower HA expression (mean for all clones, 54%) when compared with clones 10, 11, and 14 of group I (mean, 93%).

We then transfected the SP1 cell line with the murine IL-2 gene and were able to obtain SP1 cells secreting high levels of IL-2 (20000 U/ml in 72 h of culture). SP1/IL-2 cells express class II but not class I MHC antigens, and yet fail to grow in syngeneic animals but do grow in nude mice (Table 6, next page). Of interest is that these cells do not protect against a challenge with parental cells.

## Discussion

Transfection of the SP1 murine mammary adenocarcinoma with the *HA* gene results in the selection of an immunogenic SP1 population. The H4A and H4B cell lines were selected from *HA*-transfected SP1 cells by FACS using an anti-HA antibody. Both lines are immunogenic in normal mice (but grow in immunosuppressed mice) despite the fact that the cells express only low levels of class I MHC antigens. Both the *HA*-transfected and parental SP1 cells express class II antigens (I-A$^k$) and can engender a CTL response against parent SP1 cells [16, 18].

Although the expression of HA increases the immunogenicity of SP1 cells, it does not provide for protection against a challenge with non-transfected SP1 cells. It may be that class II MHC antigen expression allows for the immune rejection of HA-expressing cells by class II MHC restriction [16, 18], but this is not definitively proven in these studies because we were unable to obtain a class-II-MHC-negative control population.

We also performed experiments to assess the role of class I MHC in protection. The H5AK5 population was selected by FACS using anti-H-2K$^k$ antisera. We would emphasize again that pSV2neo transfection alone did not increase class I MHC expression. Sixty-six percent of H5AK5 cells expressed K$^k$ and were shown to protect against an SP1 challenge. The requirement for class I MHC was further shown by cloning experiments that allowed us to isolate clones that expressed high levels of HA in association with somewhat lower but adequate levels of surface

**Table 4.** Immunogenicity of H-2$^k$ and HA-expressing cells[a]

| Cell line | No of mice with tumor/ no. of mice used | | | | $10^{-3}\times LD_{50}$[b] |
|---|---|---|---|---|---|
| | Challenge dose | | | In immuno-suppressed mice ($1\times10^4$ cells) | |
| | $1\times10^4$ cells | $5\times10^4$ cells | $1\times10^5$ cells | | |
| H4A | 1/16* | 4/12* | 9/12 | 8/8 | 64 |
| H5AK5 | 0/10* | 0/ 5* | 3/10* | 7/7 | 280 |
| SP1 | 15/15 | 15/15 | 15/15 | | 1.5 |

[a] Groups of CBA mice were injected with H4A or cell lines selected for H-2 expression five times (H5AK5) and observed for tumor growth

[b] The number of tumor cells required for 50% lethal growth in syngeneic mice.

* Statistically significant compared with control SP1 cells; $P$ <0.001

**Table 5.** Immunogenicity of H5AK5 clones expressing HA and/or H-2K$^k$ antigens[a]

| H5AK5 clones | HA expression | | H-2K$^k$ expression | | No. of mice with tumor/no. challenged | | | No. of mice with growth of SP1/no. challenged ($1 \times 10^4$ cells) | *P* |
|---|---|---|---|---|---|---|---|---|---|
| | Log fluorescence | % | Log fluorescence | % | In normal mice | | In immuno-suppressed mice ($5 \times 10^5$ cells) | | |
| | | | | | $1 \times 10^5$ cells | $5 \times 10^5$ cells | | | |
| Group I | | | | | | | | | |
| Cl 10 | 132±38 | 94 | 96±30 | 68 | 1/ 6 | 7/18 | 3/ 3 | 2/ 6 | <0.01 |
| Cl 11 | 200±32 | 99 | 91±12 | 52 | 0/ 5 | 1/13 | 3/ 3 | 2/18 | <0.001 |
| Cl 14 | 133±41 | 87 | 91± 9 | 41 | 0/10 | 4/13 | | 4/ 7 | <0.05 |
| Group II | | | | | | | | | |
| Cl 1 | 87±13 | 45 | 116±12 | 96 | 4/10 | NT | | | |
| Cl 3 | 93±31 | 74 | 93±14 | 77 | 1/ 5 | 3/18 | 3/ 3 | 2/ 3 | NS |
| Cl 13 | 93±20 | 47 | 113±15 | 92 | 1/ 6 | 4/ 8 | | | |
| Cl 16 | NT | 66 | NT | 22 | NT | 5/10 | 3/ 3 | 6/ 7 | NS |
| Cl 21 | 89±18 | 39 | 99±12 | 89 | 0/10 | 4/ 8 | | 3/ 3 | NS |
| Cl 24 | 89±13 | 51 | 124±13 | 98 | 4/10 | NT | | | |
| Cl 25 | 112±42 | 74 | 88±13 | 12 | 3/11 | 6/ 8 | | | |
| Cl 26 | 82±12 | 37 | 117±13 | 99 | 4/ 6 | 8/13 | 3/ 3 | 6/ 9 | NS |
| Group III | | | | | | | | | |
| Cl 4 | 82±19 | 2 | 101±13 | 72 | 6/ 6 | 3/ 3 | | | |
| Cl 5 | 79±11 | 6 | 110±15 | 86 | 6/ 6 | 10/10 | 3/ 3 | | |
| Cl 7 | NT | 19 | NT | 46 | 4/ 6 | 8/10 | | 6/ 6 | NS |
| Cl 8 | 97±24 | 51 | 87±10 | 8 | 4/ 6 | 7/ 7 | | | |
| Cl 9 | 90±18 | 12 | 82±10 | 58 | 6/ 6 | 3/ 3 | | | |
| Cl 15 | 79±36 | 9 | <70 | 20 | 5/ 6 | 9/10 | 3/ 3 | 8/10 | NS |
| Cl 22 | 99±30 | 7 | 94±25 | 24 | 5/ 5 | 3/ 3 | | | |
| Cl 23 | NT | 7 | NT | 85 | NT | 4/ 5 | | | |
| H5AK5 parent line | 162±49 | 84 | 90± 9 | 66 | 3/15 | 7/ 9 | 8/ 8 | 4/ 8 | <0.05 |
| SP1 parent | | | <70 | 5 | 15/15 | 15/15 | 15/15 | 20/20 | |

[a] Nineteen clones derived from H5AK5 were tested for HA and H2K$^k$ expression. The clones were injected into syngeneic and immunosuppressed mice so as to assess their immunogenicity. In addition, the clones were used as immunogens to determine if they could protect against a challenge with SP1 cells. Groups of CBA mice were immunized with two injections of $1 \times 10^6$ H5AK5 or its clones given on days 0 and 14. Animals were challenged with parent cells on day 21. NT, not tested; NS, not significant

**Table 6.** Immunogenicity of -SP1/IL-2 cells in syngeneic CBA mice

| | No. of mice with tumor/ No. of mice injected Challenge dose | | | | Growth in immuno-suppressed mice |
|---|---|---|---|---|---|
| | $5 \times 10^4$ | $1 \times 10^5$ | $5 \times 10^5$ | $1 \times 10^6$ | $1 \times 10^4$ |
| SP1 | 20/20 | – | – | – | NT |
| SP1/IL2 | 0/4 | 0/4 | 0/6 | 3/15 | 4/4 |

Groups of CBA mice were injected s.c. with different doses of IL-2 transfected (and expressing) SP1 cells. In addition, 4 immunosuppressed nude mice were injected with $1 \times 10^4$ SP1/IL-2 cells. Tumor growth was assessed by observation

K$^k$. Only clones expressing high HA levels in conjunction with K$^k$ could provide significant protection against an SP1 challenge. In addition, SP1/IL-2 cells failed to grow in syngeneic animals but did not express class I MHC antigens and could not protect against a parental SP1 challenge. These latter experiments provide circumstantial evidence for the need of class I antigens in the generation of a protective immune response.

Previous reports on MHC expression by SP1 cells have produced different results based on the means used to select for MHC expression. Carlow et al. [1] originally showed that SP1 cells treated with 5-azacytidine had augmented class I MHC expression associated with their increased immunogenicity. In their hands no class II (A$^k$ or E$^k$) antigens were detected on the parent SP1 cells or the immunogenic variants [3]. However, some variants expressed high levels of class I antigens but were tumorigenic. This was confirmed by a more recent report by Carlow et al., showing that the expression of class I MHC was insufficient to confer an immune phenotype on 5-azacytidine-treated cells [2]. This is an important issue and emphasizes why we did not feel it necessary to select for class I expressing cells from SP1 cells transfected with pSV2neo alone. Since 5-azacytidine-treated cells constitutively expressed class I MHC antigens but retained their tumorigenicity it was quite clear that class I MHC antigen expression alone was insufficient to confer an immune phenotype on SP1 cells. This was further confirmed by the experiments with the SP1/IL-2 cells that express little class I antigen but are immunogenic in syngeneic animals.

The studies of Carlow et al. differ from our own in several important ways. We did not select our immunogenic variants after drug treatment, but chose instead to transfect SP1 cells with an antigen (HA) capable of induc-

ing what has been called associative recognition: the presence of an alien antigen such as HA is thought to provide a means for engendering a response to the putative tumor antigen(s) [11, 13, 17]. We were fortunate to be able to demonstrate such an effect possibly because, in contrast to Elliott et al. and Carlow et al. [1–3], the SP1 line we have maintained independently in vitro expresses $IA^k$. In agreement with these authors we could demonstrate only low levels of expression of class I antigens on SP1 cells. While our data favor the role of class II in the immune rejection of HA-transfected SP1 cells, it is possible that other as yet undefined surface antigens may provide the means for the response to HA.

The immunogenicity of HA expressing cells in normal CBA mice appears likely to be due to a class-II-mediated immune response. This was further substantiated by our ability to inhibit the H4B CTL response with anti-CD4 but not anti-CD8 antibody. This observation is in agreement with the evidence that class II restriction is effected through CD4 not CD8 cells [14, 21]. It may well be that in this tumor system the low level of class I MHC antigen expression is sufficient for a moderate CD8-mediated cytotoxic response that enhances the CD4-mediated response. The selection of the H5AK5 class-I-MHC-antigen-expressing line subsequently resulted in our ability to clone highly immunogenic variants able to induce an immune response to themselves and to provide protection against a substantial challenge with SP1 cells.

The mechanism of the protective response is not certain but we envision the following scenario. The expression of class II MHC antigen with HA is insufficient to provide a protective response against the tumor antigen expressed by SP1 cells. However, the presence of HA and class I MHC antigens provides the stimulus necessary to induce a response to HA and the SP1 tumor antigen. Such a response is now capable of recognizing the SP1 tumor antigen in conjunction with $I\text{-}A^k$, as expressed on the non-class-I, non-HA-expressing parent SP1 cells. These findings could relate to early observations relating class I MHC expression to tumor rejection [19, 20], as well as to studies demonstrating a role for class I and II MHC antigens in the immune recognition of influenza virus infections [22].

These experiments support the view that immunotherapy could be based on the enhanced expression of MHC antigens along with more immunogenic i. e., alien surface antigens. It is apparent that many tumor cells have been selected for their *inability* to express immunogenic antigens but that these antigens can be recognized if an appropriate response can be engendered. Transfection with alien genes, possibly in association with IFN$\gamma$ treatment, may provide for such a response.

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