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DIFFERENTIAL ABILITY OF TUMOR-UNIQUE AND CROSS-REACTIVE ANTIGEN(S) ON TWO MURINE HEPATOMA CELL LINES TO INDUCE Lyt-1$^+$2$^-$ T CELLS RESPONSIBLE FOR IN VIVO PROTECTIVE IMMUNITY

JUNKO SHIMA, TAKAYUKI YOSHIOKA, ATSUSHI KOSUGI MASATO OGATA, HIROMI FUJIWARA[1] and TOSHIYUKI HAMAOKA

Department of Oncogenesis, Institute for Cancer Research, Osaka University Medical School, 1–1–50, Fukushima, Fukushima-ku, Osaka 553, Japan

SHIGEHARU UEDA and SHIRO KATO

Department of Pathology, Research Institute for Microbial Diseases, Osaka University, 3–1, Yamadaoka, Suita, Osaka 565, Japan

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SUMMARY The role of the tumor-unique determinant(s) on two syngeneic murine hepatoma cells in inducing in vivo protective immunity was investigated in comparison with that of the tumor-cross-reactive determinant(s). Induction of vaccinia-reactive helper T cells in C3H/He mice by intraperitoneal (i.p.) inoculation of viable vaccinia virus and then immunization with vaccinia-infected syngeneic MH134 or MH129 tumor cells resulted in the production of potent anti-MH134 or -MH129 antibody as well as the generation of in vivo protective immunity. Neither antibody reacted with other syngeneic plasmacytoma or fibrosarcoma cells, but both cross-reacted appreciably with the other hepatoma cells as well reacted strongly as with the tumor cells used for immunization. The absorptions of anti-MH134 and -MH129 antisera with the respective hepatoma cells abolished their reactivities with both the corresponding hepatoma cells and the other hepatoma cells. In contrast, the absorption of these antisera with the other tumor cells resulted in loss of their cross-reactivities with the other hepatoma cells, but not loss of their specific reactivity to the respective hepatoma cells. Although in these hematoma systems, the above-mentioned immunization protocol resulted in in vivo indution of protective immunity and generation of antibodies, in vivo immunity as observed by Winn assays was mediated by Lyt-1$^+$2$^-$ T cells and was specific for each type of hepatoma cells. These results

1 To whom correspondence should be addressed

2 Abbreviations used in this paper: TATA, tumor-associated transplantation antigen(s); MCA, methylcholanthrene; MMC, mitomycin C; i.p., intraperitoneal or intraperitonally; FMF, flow microfluorometry; ELISA, enzyme-linked immunosorbent assay; C, complement; i.d., intradermal.

indicate that these two types of hepatoma cells bear two kinds of antigenic determinants, one kind unique to each hepatoma and the other kind cross-reactive with the other hepatoma cells. The results also indicate that none of the determinants detected by antibody are capable of inducing protective Lyt-1^+2^- T cells in vivo.

INTRODUCTION

Chemically induced mouse sarcomas possess tumor-associated transplantation antigens (TATA). Most of the TATA on these tumor cells appears to be unique to each tumor, even when the tumors are induced by the same carcinogen and exhibit the same morphology (Klein et al., 1960). However, evidence for the existence of common TATA in chemically induced mouse tumors has also been presented (Hellstrom et al., 1978). For example, common TATA have been demonstrated by cross-protection between different methylcholanthrene (MCA)-induced fibrosarcomas. Althought these studies have indicated the existence of both tumor-unique and cross-reactive antigens in chemically induced murine sarcomas, nothing is known about the structural relationship between these two types of TATA or the mechanisms responsible for the diversity of unique antigens. Studies on these problems are difficult because MCA-induced tumors usually do not generate anti-TATA antibody responses and no antibody is available for use in analyses. Thus, the development of tumor models in which TATA are capable of inducing antibody responses together with in vivo protection should be useful for studies on the above problems.

We have found that TATA on CCl_4-induced murine hepatomas can induce potent antibody together with in vivo protection (Shimizu et al., 1984; Fujiwara et al., 1986). In the present study, we examined whether two of these hepatomas express unique and cross-reactive TATA and whether both these TATA induce antibodies and in vivo protective T cell responses. We found that C3H/HeN mice immunized with syngeneic MH134 or MH129 hepatoma cells showed anti-TATA antibody responses unique to each tumor and cross-reactive with the two tumors, but not with another syngeneic tumor. In contrast, in vivo protective immunity mediated by Lyt-1^+2^-T cells was specific to the respective hepatomas, indicating the importance of tumor unique TATA in inducing in vivo protective immunity. These results are discussed in the context of the applicability of these tumor models in analyses of the immunochemical nature of the antigenic unit inducing resistance as well as the structural relationship between unique and cross-reactive TATA.

MATERIALS AND METHODS

1. *Mice and tumors.*

Female C3H/HeN mice were purchased from Charles River Laboratory, Kanagawa, Japan, and used at 7–10 weeks of age. CCl_4-induced MH134 and MH129 hepatomas derived from C3H/He strain, were utilized.

2. *Virus.*

Vaccinia virus (strain Ikeda) was propagated in chorioallantoic cavity of embryonated hens' eggs in our laboratory. The infectious titer of stock virus was usually 2×10^8 plaque forming units (PFU)/ml.

3. *Immunization to tumors.*

Immunization to the MH134 or MH129 hepatoma cells was performed according to the protocol of augmented induction of tumor-specific immunity utilizing virus-help, as described previously (Shimizu et al., 1984). Briefly, C3H/HeN mice were inoculated with 10^7 PFU of vaccinia virus to generate virus-reactive helper T cell activity and 4 weeks later immunized intraperitoneally (i.p.) with 10^7 mitomycin C (MMC)-treated vaccinia virus-infected tumor cells 4 times at 7–10 day intervals.

4 *Immunofluorescence staining and flow microfluorometry (FMF).*

The preparation and staining procedures were essentially the same as described previously (Ogata et al., 1986). Briefly, 1×10^6 tumor cells were incubated at 4 C for 30 min with anti-MH134 or -MH129 antiserum, then washed twice by centrifugation, incubated at 37 C for 30 min with fluoresceinated goat $(Fab')_2$anti-mouse IgG (Cappel Laboratories Inc., Cochranville, PA.), washed twice, resuspended and examined for fluorescence. These procedures were performed in Hanks' balanced salt solution (without phenol red) containing 0.1% bovine serum albumin (BSA) and 0.1% sodium azide. FMF analysis was performed using a Spectrum III (Ortho Diagnostic Systems Inc., Raritan, N.J.). All data were collected using log amplification and dead cells were rejected from analysis by forward light scatter.

5. *Enzyme-linked immunosorbent assay (ELISA) for measuring alloantibody activity.*

MH134 or MH129 tumor cells (C3H/He origin) suspended in 0.15 *M* Nacl, 0.2% BSA and 0.005 *M* phosphate buffer (pH 7.4) were dispensed (1×10^5/well) into a 96-well round bottom microtiter plate (Corning Glass Works, Corning, NY.) and centrifuged at $200\times g$ for 5 min. The pellet of the cells was resuspended in 50 μl of anti-MH134 or-MH129 antiserum diluted in the same buffer. After incubation for 45 min at room temperature, the cells were washed three times and resuspended in 50 μl of goat anti-mouse IgG (or IgM) coupled to horse radish peroxidase (Cooper Biomedical Inc., Malvern, PA.) at a dilution of 1 : 1200. After incubation for 30 min at room temperature, the cells were washed three times and resuspended in 100 μl of 1% 2, 2′-azino-di(3-ethylbenzthiazoline sulfonate) (Zymed Laboratories Inc., San Francisco, CA.), 0.03% H_2O_2 and 0.1 *M* citrate (pH 4.2). The reaction was then terminated by adding 100 μl of 2 m*M* sodium azide and the plate was centrifuged at $200\times g$ for 5 min. A sample of 100 μl of the supernatant removed from each well was placed into a 96-well flat bottom microtiter plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and its optical density at 415nm was measured with an Immuno Reader NJ-2000 (Japan Intermed Co., Tokyo, Japan).

6. *Tumor-neutralization test (Winn assay).*

Spleen cells ($1–2\times10^7$) from normal or tumor-immunized mice were admixed with 10^5 viable tumor cells, and inoculated i.d. in a volume of 0.1 ml into syngeneic C3H/HeN recipient mice. Tumor growth was expressed as the mean tumor diameter and standard error (S.E.) of 5 mice per group.

7. *Absorption of antiserum with tumor cells.*

Anti-MH134 or MH-129 antiserum (1.0 ml) was absorbed with either MH134 or MH129 tumor cells (10^8) twice for 45 min on ice.

8. *Treatment of immune spleen cells with antibody plus complement (C).*

Monoclonal antibodies to Thy-1.2, Lyt-1.1 and Lyt-2.1 were purchased from New England Nuclear, Boston, MA. Spleen cells (10^8) from tumor-immunized mice were incubated at room temperature for 30 min with the above monoclonal antibodies at a dilution of 1 : 1000 for anti-Thy-1.2 or 1 : 100 for anti-Lyt-1.1 and anti-Lyt-2.1 in a 1-ml volume. The cells were then washed and incubated at 37 C for 45 min with rabbit C preabsorbed with syngeneic mouse spleen cells at a final dilution of 1 : 20 (Fujiwara et al., 1984).

RESULTS

C3H/HeN mice that had been primed with vaccinia virus to generate vaccinia virus-reactive helper T cells were immunized i.p. with 10^7 vaccinia virus-infected MH134 or MH129 tumor cells (MMC-treated) 4 times at 7–10 day intervals. Seven days after the final immunization, the mice were tested for anti-tumor antibody responses by flow microfluorometry (FMF) (Fig. 1) and enzyme-linked immunosorbent assay (ELISA) (Fig. 2). The results demonstrated that immunization with MH134 or MH129 tumor cells induced a potent anti-tumor antibody response to the corresponding tumor cell types. Although neither antiserum reacted with another syngeneic X5563 plasmacytoma or methylcholanthrene (MCA)-induced fibrosarcoma cells (data not shown), they exhibited appreciable cross-reactivity with the other hepatoma cells.

For determination of whether these antisera contained antibodies against tumor-unique and cross-reactive determinants, each antiserum

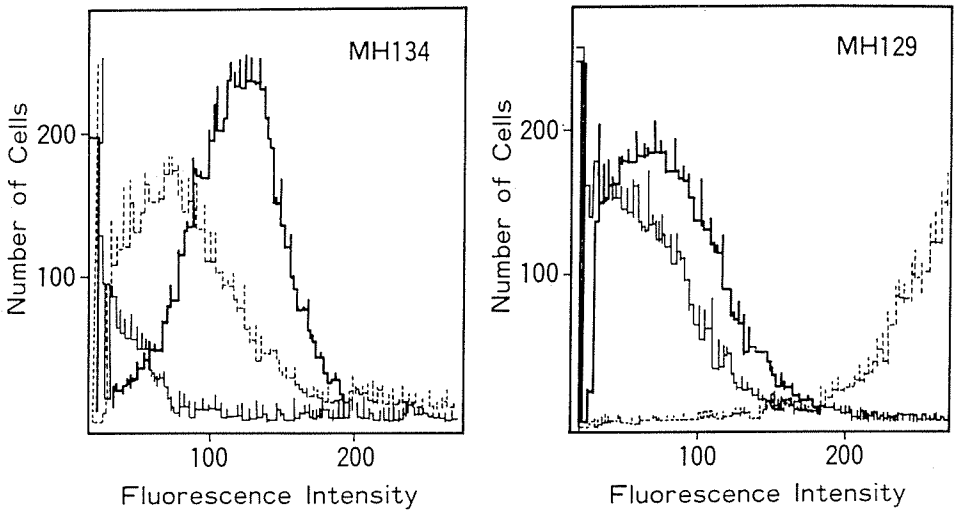

FIGURE 1. Reactivities of anti-MH134 and -MH129 antisa with the corresponding and the other tumor cells (FMF analysis). MH134 or MH129 tumor cells were stained with either normal (——), MH134-immune (——) or MH129-immune (- - -) serum followed by fluorescein-conjugated goat $(Fab')_2$ anti-mouse IgG (obtained from Cappel Laboratories Inc. Cochranville, PA).

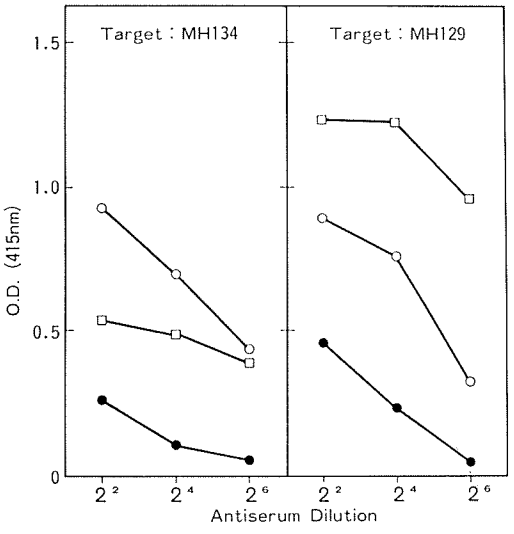

FIGURE 2. Reactivity of anti-MH134 or -MH129 antiserum with the corresponding and the other tumor cells (ELISA analysis). The anti-MH134 and -MH129 antibody activities in antisa were determined by ELISA. ●, normal serum; ○, MH134-immune antiserum; □, MH129-immune antiserum.

was absorbed with either one of the hepatoma cells (Figs. 3, 4). Absorption of anti-MH134 antiserum with MH134 tumor cells resulted in almost complete loss of reactivity with MH134 tumor cells, whereas its absorption with MH129 tumor cells did not result in loss of anti-MH134 reactivity (Fig. 3A). Portions of the same antisera absorbed with either MH134 or MH129 cells lost reactivity with MH129 tumor cells (Fig. 3B). Likewise, when anti-MH129 antiserum was absorbed with MH129 tumor cells, it lost both anti-MH129 reactivity and anti-MH134 cross-reactivity. However, anti-MH129 antiserum absorbed with MH134 tumor cells still exhibited appreciable anti-MH129 reactivity. These results indicate that each hepatoma cell type expresses tumor-unique and cross-reactive antigenic determinants and that both antisera contain antibodies to both determinants.

An earlier study has demonstrated that immunization of vaccinia-primed mice with vaccinia-infected MH134 tumor cells resulted in in vivo protective immunity along with the

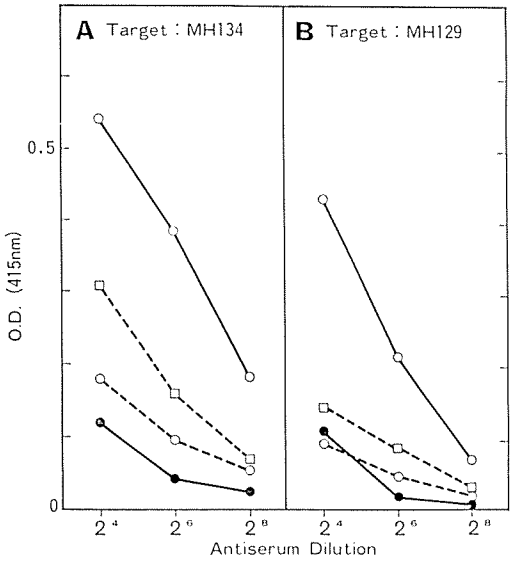

FIGURE 3. Detection of anti-MH134-specific antibody activity in MH134-immune serum. Anti-MH134 antiserum was absorbed with either MH134 or MH129 tumor cells. The reactivity of this serum without absorption (○——○) and after absorption with MH134 (○- - -○) or MH129 tumor (□- - -□) cells were determined by ELISA. Values for normal serum (●——●) are also shown.

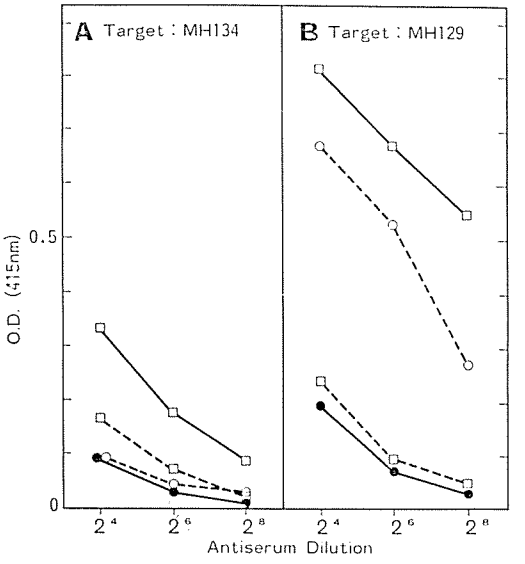

FIGURE 4. Detection of anti-MH129-specific antibody activity in MH129-immune serum. Anti-MH129 antiserum was absorbed with either MH129 or MH134 tumor cells. The reactivity of this serum without absorption (□——□) and after absorption with MH129 (□- - -□) or MH134 tumor (○- - -○) cells were determined by ELISA. Values for normal serum (●——●) are also shown.

TABLE 1. *Lyt-1$^+$2$^-$ T Cell-mediated In Vivo Protective Immunity in MH134 and MH129 Tumor Systems*

Spleen cells used in Winn assay (treatment[a])	Tumor growth (diameter in mm)	
	Day 6	Day 10
normal	3.9±0.1	8.5±0.6
MH134-immune (−)	<3.0	<3.0
MH134-immune (C)	<3.0	<3.0
MH134-immune (anti-Lyt-1.1+C)	3.5±0.2	7.3±0.5
MH134-immune (anti-Lyt-2.1+C)	<3.0	<3.0
normal	5.1±0.4	7.6±0.5
MH129-immune (−)	<3.0	<3.0
MH129-immune (C)	<3.0	<3.0
MH129-immune (anti-Lyt-1.1+C)	5.0±0.5	7.8±0.7
MH129-immune (anti-Lyt-2.1+C)	<3.0	<3.0

[a] Spleen cells were treated with monoclonal anti-Lyt-1.1 or Lyt-2.1 plus complement (C) and MH134-immune and MH129-immune spleen cells (10^7) were mixed with viable MH134 and MH129 tumor cells, respectively, for Winn assays.

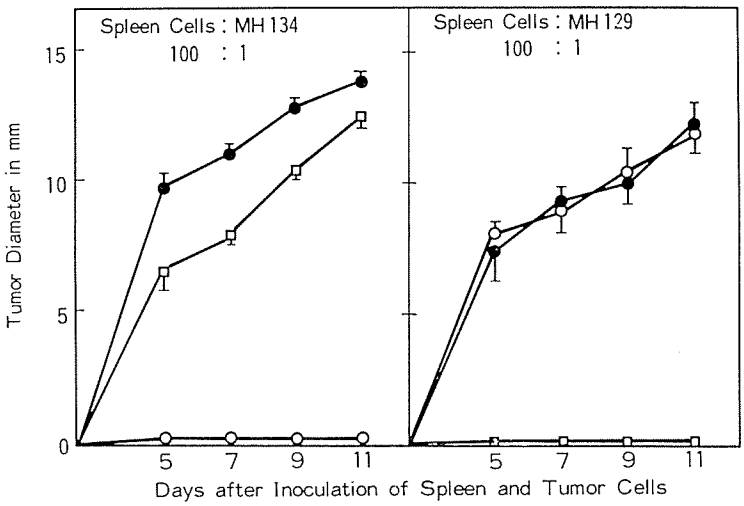

FIGURE 5. Specificity of in vivo tumor-neutralizing activity in MH134- or MH129- immune spleen cells. Spleen cells (10^7) from either normal (●—●), MH134-immune (○—○) or MH129-immune (□—□) mice were mixed with viable MH134 or MH129 tumor cells (10^5) and the mixtures were inoculated i.d. into normal syngeneic C3H/HeN recipient mice.

induction of anti-MH134 antibody activity. The results in Table 1 confirm the induction of in vivo protective immunity with a vaccinia-helper system in both hepatoma models and demonstrate the mediation of these in vivo immunities by Lyt-1^+2^-immune T cells. An additional experiment was performed to investigate whether Lyt-1^+2^- T cell-mediated in vivo protective immunity is hepatoma-specific or cross-reactive. The results in Fig. 5 demonstrate that the in vivo protective immunity as detected by Winn assays is strictly specific for each hepatoma. Although appreciable cross-reactivity was observed in the anti-tumor antibody responses, no cross-protective element was detected even when Winn assay was performed at a higher ratio of spleen to tumor cells (300: 1) (data not shown).

DISCUSSION

A striking feature of the TATA of chemically induced murine tumors is their polymorphism, each neoplasm having a TATA unique to itself (Klein et al., 1960). This has been a common finding over the past 25 years, but recent investigations have revealed common or restricted cross-reactive TATA in chemical and/or ultraviolet-induced murine sarcomas (Hellstrom et al., 1978; Ransom et al., 1981).

Earlier studies from our laboratory have demonstrated that preinducing vaccinia virus-reactive helper T cells by i.p. inoculation of viable virus and subsequently immunizing with vaccinia virus-infected syngeneic tumor cells resulted in anti-tumor cytotoxic T lymphocyte (CTL) and/or antibody responses along with the induction of in vivo resistance (Shimizu et al., 1984). It should be noted that such an immunization protocol produced an antibody response in CCl_4-induced murine hepatoma models, although any antibody activity was not detected even by the above immunization procedure in most of other syngeneic tumor models including MCA-induced sarcomas. Thus, by studies on the anti-hepatoma antibodies in murine hepatoma models, the existence of tumor-unique and cross-reactive determinants could be demonstrated and their possible relation to the determinants responsible for induc-

ing in vivo protective immune resistance could be analyzed.

In the present study using anti-tumor antibodies, we demonstrated the existence of tumor-unique and cross-reactive antigenic determinants in two CCl_4-induced murine hepatomas cell lines, MH134 and MH129. However, as with most of chemically induced tumor models, the natures of these two types of antigens could be determined. Murine leukemia virus (MuLV) antigens are commonly associated with murine tumor cells. In fact, Zbar et al. (1981) have presented evidence that the antigens coded for by an endogenous MuLV function as the common (cross-reactive) TATA on chemically induced sarcomas, although restricted cross-reactivity for MuLV-unrelated antigens is also seen in some sarcoma models. Since both MH134 and MH129 hepatoma cells reacted with anti-MuLV-determined gp 70 antibody (unpublished observations), the cross-reactive determinants detected with anti-MH134 or -MH129 antibody may represent MuLV-related antigens.

Irrespective of whether the cross-reactive determinants on MH134 and MH129 cells are related to MuLV-related antigens, these antigens played only a marginal role in inducing in vivo resistance. In contrast, studies on absorption of anti-MH134 or -MH129 antibody revealed the existence of tumor-unique determinants on each hepatoma. More importantly, Lyt-1^+2^- T cell-mediated in vivo protective immunity was hepatoma-specific. These findings confirm the importance of tumor-unique determinants in induction of in vivo protection, although the interrelationship between tumor-unique determinants as detected by antibody and determinants able to induce Lyt-1^+2^- T cells remain to be further investigated.

The central issue in further studies on tumor antigens should be the immunochemical nature of tumor-unique TATA and the molecular mechanism involved in generation of diversity of unique TATA. One approach would be to solubilize and isolate TATA molecules from tumor cell membranes. Studies along these lines are now in progress in our laboratories using the antibodies against tumor-unique determinants on hepatoma cells that are now available and some monoclonal antibodies that have recently been established.

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REFERENCES

Fujiwara, H., Fukuzawa, M., Yoshioka, T., Nakajima, H., Hamaoka, T. 1984. The role of tumor-specific Lyt-1^+2^- T cells in eradicating tumor cells in vivo. I. Lyt-1^+2^- T cells do not necessarily require recruitment of host's cytotoxic T cell precursors for implementation of in vivo immunity. J. Immunol. 133: 1671–1676.

Fujiwara, H., Yoshioka, T., Shima, J., Kosugi, A., Itoh, K., Hamaoka, T. 1986. Helper T cells against tumor-associated antigens (TATA): Preferential induction of helper T cell activities involved in anti-TAA cytotoxic T lymphocyte and antibody responses. J. Immunol. 136: 2715–2719.

Hellstrom, K. E., Hellstrom, I., Brown, J. P. 1978. Unique and common tumor-specific transplantation antigens of chemically induced mouse sarcomas. Int. J. Cancer 21: 317–322.

Klein, G., Sjogren, H. O., Klein, E., Hellstrom, K. E. 1960. Demonstration of resistance against methylcholanthrene induced sarcomas in the primary autochthonous host. Cancer Res. 20: 1561–1572.

Ogata, M., Shimizu, J., Tsuchida, T., Takai, Y., Fujiwara, H., Hamaoka, T. 1986. Non-H-2-linked genetic regulation of cytotoxic responses to hapten-modified syngeneic cells. I. Non-H-2-linked Ir gene defect expressed on T cells is not predetermined at the stage of bone marrow cells.

J. Immunol. 136: 1178–1185.
Ransom, J. H., Schengrund, C-L., Bartlett, G. L. 1981. Solubilization and partial characterization of a tumor-rejection antigen from an ultraviolet light-induced murine tumor. Int. J. Cancer 27: 545–554.
Shimizu, Y., Fujiwara, H., Ueda, S., Wakamiya, N., Kato, S., Hamaoka, T. 1984. The augmentation of tumor-specific immunity by virus-help. II. Enhanced induction of cytotoxic T lymphocyte and antibody responses to tumor antigens by vaccinia-reactive helper T cells. Eur. J. Immunol. 14: 839–843.
Zbra, B., Manohar, V., Sugimoto, T., Ashley, M. P., Kato, Y., Rappaport, P. 1981. Immunoprophylaxis of transplantable MC-induced murine fibrosarcomas by immunization with embryo cells expressing endogenous murine leukemia virus antigens. Cancer Res. 41: 4499–4507.