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P815 Tumor Cells Express an Intrinsic Absolute Dominant Tumor Specific Transplantation Antigen (TSTA) That Functions in Immune Regulation

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Key words : minor histocompatibility antigen, tumor specific transplantation antigen, cytotoxic T lymphocytes, bone marrow cells, dominant antigen

ABSTRACT

As we have reported[1], Thy.1$^-$ bone marrow cells passed through nylon wool (NW-BM) augmented the generation of cytotoxic T lymphocytes (CTL) against minor histocompatibility (mH) antigens in primary mixed lymphocyte cultures (MLC). This study showed that when DBA/2 (H-2^d) responder lymph node (LN) cells were stimulated with syngeneic P815 (H-2^d, DBA/2 origin) tumor cells in the presence of DBA/2 helper NW-BM cells, the CTL specific for the tumor specific transplantation antigen (TSTA) of P815 tumor cells were induced easily in primary cultures *in vitro*.

We also showed that the CTL from Balb/c (H-2^d) or B10D2 (H-2^d) responder cells stimulated with DBA/2 spleen cells killed DBA/2 Con A blast cells but not P815 tumor cells. Since the CTL recognized the absolute dominant mH antigen of DBA/2 lymphoid cells according to the previous report[1], P815 tumor cells had lost this antigen. On the other hand, the CTL from Balb/c or B10D2 responder cells stimulated with allogeneic P815 tumor cells lysed P815, but not DBA/2 target cells.

Thus, P815 tumor cells expressed the intrinsic absolute dominant antigen, which was absent in DBA/2 lymphoid cells.

INTRODUCTION

The frequency of tumor incidence is repressed by immune surveillance[2]. It was thought that T cells, natural killer (NK) cells, lymphokine activated killer (LAK) cells and macrophages contributed to this effect. If NK cells played the major role in immune surveillance, the tumor cells should become NK-resistant since they progressed by overcoming the selection by NK cells *in vivo*. In particular, the progressor variants that arose spontaneously from regressor fibrosarcoma cells which were induced in mice by ultraviolet radiation, were NK sensitive, indicating that NK cells did not have the major role in immune surveillance[3]. Peripheral blood cells activated with IL-2 *in vitro* generate LAK cells[4]. Cancer cells seem to be universally susceptible to killing by LAK cells *in vitro*. Thus, LAK cells did not act as selective pressure against tumor progression. A tumor necrosis factor produced by macrophages mediates their tumoricidal activity[5]. Since a normal host could kill inoculated cancer cells which were resistant to activated macrophages[6], macrophages were not major participants in immune surveillance.

The T cell mediated immune response, which plays an important role in the protection of humans and animals against virally induced or virus associated malignancies[7,8], does not protect against spontaneous and chemical- or physical-carcinogen induced progressive tumors in normal mice[7,9]. Although the mechanisms that protect against such tumors remain largely unknown, it cannot be ruled out that these tumor cells have active antigens that stimulate the T cell mediated immune response. Evidence for the existence of tumor specific antigens on murine tumors was initially shown by the acquisition of resistance to syngeneic or auto methylcholanthrene-induced tumors following immunization with these tumor cells[10,11]. Later, similar antigens were proven for some spontaneous and some physical-carcinogen induced tumors[12,13].

In this manuscript, we reported that Thy.1$^-$ NW-BM cells helped to induce the CTL from DBA/2 (H-2^d) responder cells, which were specific for the tumor specific transplantation antigen (TSTA), in primary cultures by the stimulation with syngeneic P815 (H-2^d, DBA/2 origin) tumor cells. We also showed that the CTL from Balb/c (H-2^d) or B10D2 (H-2^d) responder cells stimulated with allogeneic P815 tumor cells killed P815 tumor cells but not DBA/2 Con A blast cells even if DBA/2 lymphoid cells and P815 tumor cells had many mH antigens in common. On the contrary, when stimulated with DBA/2 spleen cells, the CTL from Balb/c or B10D2 responder cells killed DBA/2 Con A blast cells but not P815. Thus, these data suggested according to the previous report[1] that P815 tumor cells lost the absolute dominant mH antigen expressed on DBA/2 lymphoid cells and possessed an intrinsic absolute dominant antigen that was not present on the DBA/2 lymphoid cells. We discuss the role of this antigen in regard to the regulation of tumor immunity.

MATERIALS AND METHODS

Mice

We used 8- to 16-week-old male and female mice of inbred strains, B10BR (H-2^k), B10D2 (H-2^d), Balb/c (H-2^d) and DBA/2 (H-2^d), bred in the animal facility at the School of Medicine, University of the Ryukyus, Okinawa, Japan.

Tumor cells

Methylcholanthrene induced mastocytoma, P815, originated from DBA/2 mice were used. Raji cells, which were natural killer cell resistant and lymphokine activated killer cell sensitive, were also used.

Mixed lymphocyte cultures (MLC)

The procedures are described elsewhere in detail[1]. In brief, cells from lymph nodes (LN) (inguinal, axillary, and brachial) or spleens were suspended in standard culture medium (α -minimum essential medium [α -MEM]) containing 10% fetal calf serum (FCS), 10 mM HEPES buffer, and $5x10^{-5}$ M 2-mercaptoethanol. Femur bone marrow (BM) cell suspensions were obtained by passing them through syringe needles. Two milliliters ($2.5x10^7$ cells/ml) of BM cell suspensions were loaded onto a nylon wool column. After incubation for 45 minutes at 37℃ nonadherent cells were eluted, washed once by centrifugation, resuspended in the culture medium as described previously[14] and then referred to as NW-BM cells. Several doses of LN cells (responder cells), NW-BM cells (helper cells[1]), and mitomycin-c treated DBA/2 spleen cells ($3x10^5$ cells/well) or P815 mastocytoma cells ($3x10^4$ cells/well) (stimulator cells) were co-cultured in microculture wells containing 0.2 ml of culture medium supplemented with several doses of rat T cell growth factor (TCGF) as described previously[1]. Control cultures, from which the spontaneous value was obtained, did not contain responder LN cells. These cultures were incubated for 5 days at 37℃ in an atmosphere of 5% CO_2 in air.

Antiserum

Cells were incubated with anti-Thy.1.2 or anti-Lyt.2 monoclonal antibody for 30 minutes at 4℃, washed once with cytotoxicity medium (Cedarlane Laboratories), and incubated in a water bath with rabbit complement (Cedarlane Laboratories), at a final dilution of 1:10, for 45 minutes at 37℃. Cells were then washed three times with culture medium.

Cytotoxicity assay

After five days in culture, MLC were assayed for cytotoxic activity using ^{51}Cr-labelled concanavalin A (Con A) blast target spleen cells ($1x10^3$ cells/well) or tumor cells ($1x10^3$ cells/well), as previously described[15]. The contents of each microculture well were divided into two portions. Target cells from stimulator strains were added to one well, and different target cells, not from the stimulator strains, were added to the other. The purpose of these studies was to test whether the CTL were specific only for the stimulator. After four hours in culture, specific ^{51}Cr release, p, was defined as observed counts minus spontaneous counts / total releasable counts minus spontaneous counts. Each experimental group contained 32 replicate cultures. Test wells were scored as positive if their counts were greater than the mean spontaneous value by more than 2 standard deviations ($p<0.05$).

RESULTS

The induction of cytotoxic T lymphocytes (CTL) specific for minor histocompatibility (mH) antigens

A limited number of LN cells from DBA/2 mice (H-2^d, DBA/2 background) as responder cells were cultured with mitomycin-c treated spleen cells from B10D2 mice (H-2^d, B10 background) as stimulator cells. An excess of NW-BM cells, treated with anti-Thy.1.2 monoclonal antibody and complement, from DBA/2 mice as helper cells and a minimum required dose of TCGF were also added and incubated for five days in microculture wells. In these cultures responder LN cells were so limited in number that helper T cells were almost completely diluted out and could not make contact with CTL precursors. In most experiments in this report, control cultures which contained no NW-BM cells were prepared. In those cultures no CTL was induced from responder LN cells (data not shown). As reported previously[16], substituting for helper T cells, NW-BM cells augmented the generation of allo major histocompatibility com-

plex (MHC) specific CTL. We determined whether the NW-BM cells could help the generation of CTL specific for mH antigens.

Cells in each well were divided into two at five days of culture. Into one well B10D2 target cells, identical to stimulator cells, were added. Into the other, Balb/c (H-2^d, Balb/c background) target cells were added. The CTL were induced easily in primary cultures *in vitro* and they were specific for the mH antigens (Fig. 1).

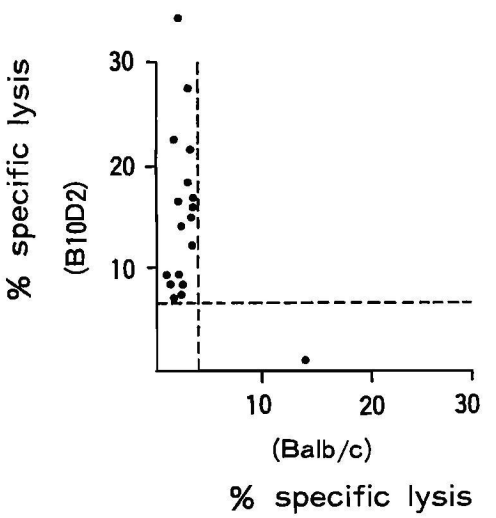

Fig. 1. The mH antigen specific CTL were induced by the helper effect of NW-BM cells in primary cultures *in vitro*. DBA/2 LN cells ($3x10^3$ cells/well), mitomycin-c treated B10D2 spleen cells ($3x10^5$ cells/well) and DBA/2 Thy.1^- NW-BM cells ($3x10^4$ cells/well) were co-cultured in 32 replicate culture wells supplemented with 0.33 units/ml of rat TCGF. Duplicate sets of these cultures were prepared. In one set, each well was split into two wells after 5 days in culture, then B10D2 Con A blast target cells were added to one well and Balb/c Con A blast target cells were added to the other. Only the positive wells were plotted according to MATERIALS AND METHODS. The broken lines show two standard deviations ($p<0.05$) of the spontaneous lysis value. In the other set of cultures (parallel cultures) B10BR target cells were added, but no lysis was detected in any wells.

Parallel cultures (32 wells) were performed with the same protocol. The CTL induced in these cultures did not lyse B10BR (H-2^k, B10 background) target cells as described in Fig. 1. Thus, CTL recognized the mH antigen restricted by self-MHC. The CTL were detected in 17 out of 32 wells and they lysed B10D2 but not Balb/c target cells. These results coincided with those reported elsewhere[1]. According to that report, these CTL recognized only the X-chromosome linked gene product, the Xir antigen, from B10D2 mice and ignored all other mH antigens. Thus, the Xir antigen was the absolute dominant antigen because it was the only stimulus despite stimulation across broad non-H-2-coded genetic differences. In these cultures inducing mH specific CTL, NW-BM cells also acted as helper cells, as reported previously for allo-MHC specific CTL induction[16].

Anomalous CTL were induced in one out of 32 wells. These CTL were anomalous killer cells since they killed third party Balb/c but not B10D2 target cells. This could be explained if the CTL were specific to the common mH antigens in both strains. However, they recognized the mH antigen that was absent in stimulator cells.

The induction of CTL specific for tumor specific transplantation antigen (TSTA)

We studied whether the CTL specific for TSTA could be induced in primary cultures *in vitro*, using the method shown in Fig. 1. A limited number of DBA/2 LN responder cells were cultured with mitomycin-c treated P815 tumor cells (H-2^d, DBA/2 origin) as stimulators.

Thy.1⁻ NW-BM helper cells from DBA/2 mice and a minimum required dose of TCGF were also added at the initiation of cultures.

After five days of culture, cytolytic activity was tested against both DBA/2 Con A blasts and P815 tumor target cells. The CTL specific for the self tumor, P815, were induced easily in our primary cultures *in vitro*. About 30% of the wells were positive for cytolysis (Fig. 2).

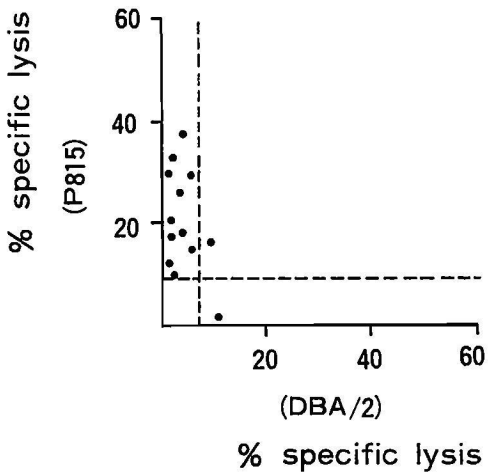

Fig. 2. The CTL specific to the TSTA of P815 tumor cells were induced in primary culture *in vitro*. The experiment was carried out the same as for Fig. 1. DBA/2 LN cells ($3x10^3$ cells/well), mitomycin-c treated P815 tumor cells ($3x10^4$ cells/well) and DBA/2 Thy.1⁻ NW-BM cells ($3x10^4$ cells/well) were co-cultured. Wells were supplemented with 0.25 units/ml of rat TCGF. P815 tumor and DBA/2 Con A blast cells were used as target cells, and positive wells were plotted.

There was, however, the possibility that P815 tumor cells were lysed by non-specific killer cells induced in these cultures, such as NK cells or LAK cells. As P815 tumor cells were NK resistant but LAK sensitive[17], the possibility of cytolysis by NK cells was negated. Cytolysis by LAK cells was excluded because LAK sensitive Raji target cells were not killed by the CTL which were specific to P815 tumor cells in 7 out of 32 wells (Fig. 4) and because Balb/c Con A blasts, that were sensitive to LAK cells, were not killed by these CTL (Fig. 3).

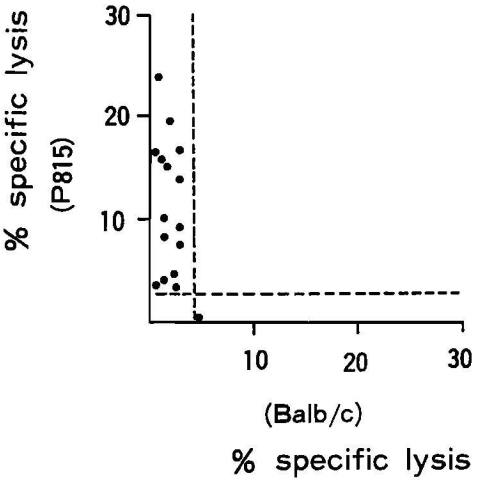

Fig. 3. The CTL specific to the TSTA of P815 tumor cells did not lyse Balb/c Con A blast target cells, which were NK resistant and LAK sensitive. Experimental procedures are described in the legend to Fig. 1. DBA/2 LN cells ($3x10^3$ cells/well), mitomycin-c treated P815 tumor cells ($3x10^4$ cells/well) and DBA/2 Thy.1⁻ NW-BM cells ($3x10^4$ cells/well) were co-cultured and supplemented with 0.33 units/ml of rat TCGF. P815 tumor and Balb/c Con A blast cells were used as target cells, and positive wells were plotted.

P815 tumor cells lost the absolute dominant antigen expressed on DBA/2 lymphoid cells

As shown in Fig. 1, the mH specific CTL were easily induced in these culture systems. We next examined the presence of mH antigens

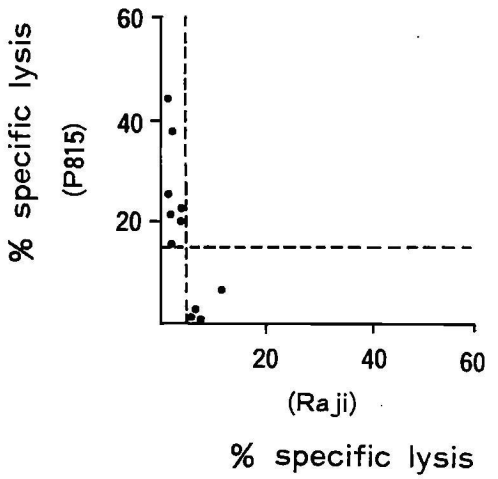

Fig. 4. The CTL specific to the TSTA of P815 tumor cells did not lyse Raji cells, which were NK resistant and LAK sensitive. The experiment was performed as described in the legend to Fig. 1. DBA/2 LN cells ($3x10^3$ cells/well), mitomycin-c treated P815 tumor cells ($3x10^4$ cells/well) and DBA/2 Thy.1$^-$ NW-BM cells ($3x10^4$ cells/well) were co-cultured and supplemented with 0.25 units/ml of rat TCGF. P815 tumor cells and Raji cells were used as target cells.

on the tumor cells. When responder Balb/c LN and helper Balb/c NW-BM cells were stimulated with spleen cells from DBA/2 mice, the induced CTL lysed DBA/2 Con A blasts but not P815 tumor target cells (Fig. 5A). Since the P815 tumor was a mastocytoma originated from DBA/2 mice, DBA/2 lymphoid cells and P815 tumor cells were known to have many mH antigens in common[18,19]. Nevertheless, these CTL did not lyse P815 tumor target cells. Elsewhere, we have reported that these CTL recognized only the absolute dominant antigen of DBA/2 lymphoid cells, and that this antigen was the only stimulus among many mH antigens in these culture systems[1]. In other words, this antigen was objective for self-nonself discri-

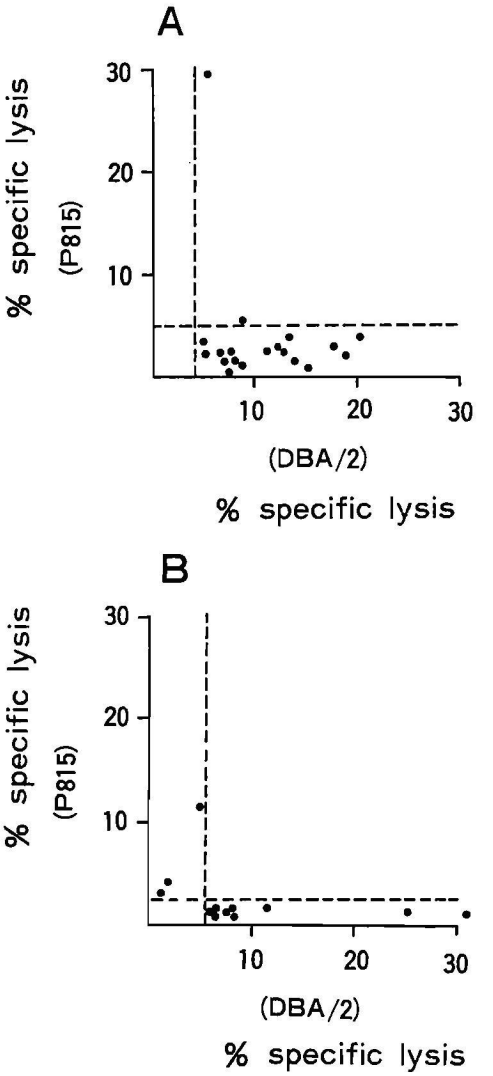

Fig. 5. P815 tumor cells lost the absolute dominant mH antigen expressed on DBA/2 lymphoid cells. The experiments were performed as described in the legend to Fig.1. Three thousands cells/well of LN and $3x10^4$ cells/well of Thy.1$^-$ NW-BM cells from Balb/c (A) or B10D2 (B) mice were co-cultured with $3x10^5$ cells/well of mitomycin-c treated DBA/2 spleen cells. Wells were supplemented with 0.33 units/ml (A) or 0.4 units/ml (B) of rat TCGF. P815 tumor and DBA/2 Con A blast cells were used as target cells.

mination and had the role of immune regulation. Thus, in these culture systems this dominant mH antigen was the most important among many. Though this antigen was expressed on DBA/2 lymphoid cells, it was not present on the P815 tumor cells originated from DBA/2 mice.

As shown in Fig. 5B, the same results as those shown in Fig. 5A were obtained when the responder and helper NW-BM cells from Balb/c mice were replaced by those from B10D2 mice and stimulated with DBA/2 spleen cells. The responses in Fig. 5B were lower than those in Fig. 5A. The responses in at least 3 out of 9 positive wells were, however, certainly significant and these wells with significant positive responses were reproduced in every other experiments (data not shown). This result showed that B10D2 responder cells might have a lower frequency of precursors of CTL specific for the DBA/2 dominant mH antigen than Balb/c responder cells.

P815 tumor cells expressed a novel absolute dominant antigen that was not seen in DBA/2 lymphoid cells

We studied whether P815 tumor cells had an absolute dominant antigen which differed from that of DBA/2 lymphoid cells. When responder Balb/c LN and helper Balb/c NW-BM cells were stimulated with P815, the induced CTL, in about two thirds of 32 wells, lysed P815 tumor cells alone but not DBA/2 Con A blast target cells (Fig. 6A). For the same reasons explained in Figs. 5A and 5B, these CTL recognized the absolute dominant antigen of P815 tumor cells. Therefore, P815 tumor cells had an intrinsic absolute dominant antigen which differed from that of DBA/2 lymphoid cells. These results showed that P815 tumor cells also had an important antigen for self-non-self discrimination and for immune regulation of CTL generation in this culture system.

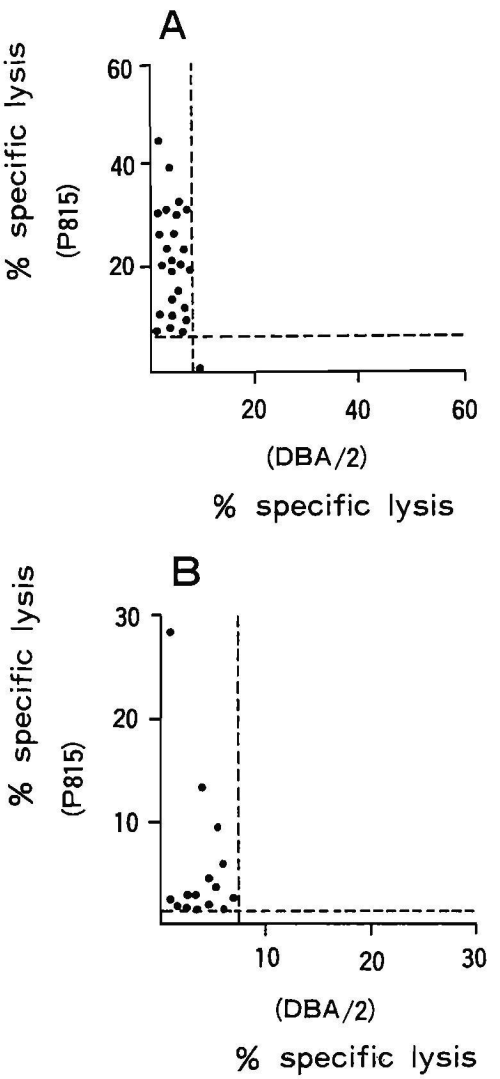

Fig. 6. P815 tumor cells expressed a new absolute dominant mH antigen, not seen in DBA/2 lymphoid cells. The experiment was carried out as described in the legend to Fig. 1. Three thousands cells/well of LN and $3x10^4$ cells/well of Thy.1$^-$ NW-BM cells from Balb/c (A) or B10D2 (B) mice were co-cultured with $3x10^5$ cells/well of mitomycin-c treated P815 tumor cells. Wells were supplemented with 0.4 units/ml (A and B) of rat TCGF. P815 tumor and DBA/2 Con A blast cells were used as target cells.

If responder LN and helper NW-BM cells, derived from B10D2 mice, were cultured with P815 tumor stimulator cells, the CTL were induced in 15 out of 32 wells (Fig. 6B). These CTL had specificity for the absolute dominant antigen of P815 tumor cells as shown in Fig. 6A. B10D2 responder cells had a lower frequency of precursors of CTL, which were specific for the absolute dominant antigen of P815 tumor cells, than Balb/c responder cells. Responses in at least 6 out of 15 positive wells were, however, certainly significant because their lysis was greater than the mean spontaneous value by more than 3 standard deviations ($p<0.01$).

DISCUSSION

When Thy.1$^-$ NW-BM were used as helper cells, CTL specific for mH antigens were generated by stimulating across broad non-H-2-coded genetic differences in primary MLC (Fig. 1). This result was the same as shown in the previous report[1], but greater responses were detected than in the previous one because NW-BM cells were treated with a lower dose of anti-Thy.1 monoclonal antibody than in the previous case. The previous report suggested that these CTL had a strict preference for the X-chromosome-linked gene product (Xir antigen), restricted to the MHC. These results suggested that the Xir antigen was the absolute dominant mH antigen in this culture system, and that it was the object for self-nonself discrimination and regulated immune response.

We studied whether tumor cells also had the absolute dominant antigen as detected in normal antigenpresentingcells (APC) of the spleen. The CTL induced from LN cells of B10D2 or Balb/c mice by stimulation with DBA/2 mitomycin-c treated spleen cells had a strict preference for DBA/2 Con A blast target cells but not for P815 tumor cells (Figs. 5A and 5B). P815 tumor cells possessed many mH antigens in common with DBA/2 lymphoid cells[18,19] because P815 tumor cells originated from DBA/2 mice that had received methylcholanthrene. Thus, we concluded that the induced CTL recognized only the absolute dominant antigen of DBA/2 lymphoid cells. It was of note that P815 tumor cells did not express the absolute dominant antigen found in DBA/2 lymphoid cells. On the contrary, P815 tumor cells possessed an intrinsic absolute dominant antigen that was not expressed on the surface of DBA/2 lymphoid cells (Figs. 6A and 6B).

TSTA of oncovirus induced tumor cells are viral gene-coded proteins[20~22]. The origin of TSTA on spontaneous and physically or chemically induced tumors is as yet unknown[23]. There are three possibilities. 1) Mutation; most, if not all, carcinogens are mutagenic[24,25]; 2) The expression of silent genes which code for the differentiation antigens, for example, TL antigens of leukemia cells, fetal antigen and cellular glycoprotein, gp70[26~29]; 3) Clonal expansion of a minor cell population which expresses a normal unique antigen that was not generally found[30]. The same issues regarding the absolute dominant antigen of P815 tumor cells are also relevant; whether the P815 dominant antigen is a mutated variant of DBA/2 dominant antigen, the result of the expression of silent gene products by which the DBA/2 dominant antigen is replaced or the normal dominant self antigen expressed on mastocyte but not lymphoid lineage. The third possibility could be neglected because the P815 absolute dominant antigen was the TSTA of P815 tumor cells which was recognized by DBA/2 responder cells as a nonself antigen (Figs. 2, 3 and 4), as discussed in detail in below. Until the P815 dominant antigen and the gene was defined, it was not determined which of the remaining possibilities were likely.

The CTL specific for the TSTA of P815

tumors were easily induced from responder DBA/2 LN cells in this primary culture system (Fig. 2). Their killing activities against P815 tumor target cells were due to neither NK nor LAK cells because P815 cells were NK resistant[17]. Also, Balb/c Con A blast target cells and Raji cells, which were NK resistant and LAK sensitive, were not killed by these induced killer cells (Figs. 3 and 4). If responder LN cells were treated with the anti-Lyt.2 monoclonal antibody and complement, no killer cell was induced in this culture system[16]. As shown elsewhere and in Fig. 1, the generation of CTL was governed only by an absolute dominant antigen, such as the Xir antigen, in this culture system[1]. Furthermore, P815 tumor cells expressed a different dominant antigen from DBA/2 lymphoid cells (Figs. 5A, 5B, 6A and 6B). Therefore, the TSTA of P815 tumor cells was identical to the P815 absolute dominant antigen because DBA/2 responder LN cells could be stimulated only by the P815 dominant antigen.

In general, the CTL specific for mH antigens were induced by priming *in vivo* and boosting *in vitro* across broad non-H-2-coded genetic differences[31]. Despite stimulation across many disparate mH antigens, the induced CTL were specific to a few, "relative" dominant mH antigens[32,33]. Thus, there was a hierarchy among the many mH antigens[34]. However, the mH antigen recognized by the CTL induced in our culture system was the "absolute" dominant single antigen, as discussed elsewhere[1]. On the other hand, the CTL induced by hyperimmunization with some tumor cells recognized the dominant tumor specific antigen among the multiple and independent tumor specific antigens[35,36]. Thus, there was an order of recognition among these tumor specific antigens[37]. Whether the "dominant tumor specific antigen" described in those reports behaves as the "dominant mH antigen" when these tumor cells are used as stimulators of CTL induction against the "mH antigen" of the tumor, remains unknown.

The TSTA is the only known variant that is restricted to tumor cells. The study of the TSTA might lead to elucidating not only the mechanism of oncogenesis but also of immune surveillance as well as how tumor cells escape it. It was therefore of interest to study whether the TSTA, or absolute dominant antigen, of P815 tumor cells defined in this culture system contributed to the malignancy of tumor cells. The large T antigens (LT) of SV-40 viruses tumorigenize cells and maintain malignancy[38]. The LT antigens are expressed on the tumor cell surface and it is the target antigen recognized by CTL, that is, TSTA[39,40] Whether LT antigens expressed on the surface have a role in the maintenance of malignancy or are simply coincidental, remains unknown. We are now studying whether LT antigens of cells transformed by SV-40 viruses act as the absolute dominant antigen. Furthermore, there is a hypothesis that TSTA are mutated cell-to-cell interaction substances related to the differentiation of cells[41]. As the P815 absolute dominant antigen, TSTA, contributed to immune regulation, it is possible that the P815 absolute dominant antigen may be such a substance, and that it induces immune suppression which allows tumor cells to escape the immune responses *in vivo*. Incidentally, it has been reported that the regulation of immune responses against the tumor antigens depends upon non-MHC[42] as well as MHC antigens[43,44] using leukemia cells induced by the murine leukemia virus (MuLV). In this report, we showed that the tumor absolute dominant antigen, TSTA, was indeed the immune regulatory gene product and was the non-MHC antigen.

It is of note that this paper reports on the intrinsic absolute dominant antigen, TSTA, of P815 tumor cells, but not of other tumor cells

so far at all. It was necessary for the extensions of this conclusion, as a general concept on tumor cells, to accumulate the studies of various other tumors.

It has been reported in detail that immune therapy against P815 solid tumor cells is successful if spleen cells from mice immunized by the irradiated P815 tumor cells are adoptively transferred into tumor bearing mice[45]. We intend to examine whether the CTL induced in the culture system used here produce a good therapeutic result when they are transferred adoptively into P815 tumor bearing mice.

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