# REGULATORY FUNCTIONS OF HAPTEN-REACTIVE HELPER AND SUPPRESSOR T LYMPHOCYTES

## III. Amplification of a Generation of Tumor-Specific Killer T-Lymphocyte Activities by Suppressor T-Cell-Depleted Hapten-Reactive T Lymphocytes*

By TOSHIYUKI HAMAOKA, HIROMI FUJIWARA, KUMIKO TESHIMA, HISAKAZU AOKI, HIROSHI YAMAMOTO, and MASAYASU KITAGAWA‡

*From the Department of Oncogenesis, Institute for Cancer Research, Osaka University Medical School, Fukushimaku, Osaka, Japan*

Tumor cells in humans (1) and in experimental animals (2) possess unique antigens, called tumor-associated transplantation antigens (TATA)[1], to which tumor-bearing hosts respond. Individuals do not promptly and consistently reject their malignant tumor cells because TATA are relatively weak immunogens. Therefore, methods to increase the stimulatory capacity are being sought.

Previously, it was demonstrated in our laboratory (3) that relatively weak immunogenic syngeneic plasmacytoma X5563 in C3H/He exclusively generated killer T-cell activity without inducing any significant helper T-cell activity against TATA. The failure to induce significant helper T-cell activity to TATA of X5563 in syngeneic system seems to be of vital importance in the following aspects. First, the X5563 syngeneic tumor system fails to provide double defense mechanisms of both cell-mediated and humoral immunity relating to the killer and helper T-cell generations, in sharp contrast to the allogeneic transplantation immunity. Furthermore, a failure to induce helper T-cell activity may have some relation to the lower level of generation of killer T-cell activity to syngeneic tumor cells in general. Cantor and Boyse (4, 5) reported that killer T-cell development against allogeneic tumor cells was augmented by the collaboration with other T-cell amplifier subsets which makes the Ly-specificity different from the killer T cells, and recognizes alloantigens other than those recognized by prekiller T cells. Although the above selective generation of killer T cells against TATA indicates that the generation of helper T-cell activity against TATA may not be an absolute prerequisite for the effective development of killer T-cell responses, it is highly conceivable that if the collaborative response of helper (amplifier) T cells with the prekiller cells can be successfully induced in syngeneic tumor system, the T-cell response against killer determinant may be augmented. In this context, if animals are immunized with tumor cells modified by additional antigenic determinants with which the helper (amplifier) T cells may be capable of reacting, the augmentation of specific killer T-cell response will be definitely expected.

In the preceding experiments, a system was established to raise monospecific hapten-reactive T-lymphocyte activity, which enabled us to understand the basic mechanisms of regulatory T-

[1] Abbreviations used in this paper: ATS, anti-thymocyte serum; B cells, precursors of antibody-forming cells; BPO, benzylpenicilloyl; CFA, complete Freund's adjuvant; D-GL, copolymer of D-glutamic acid and D-lysine; DNP, dinitrophenyl; FCS, fetal calf serum; HGG, human gamma globulin; KLH, keyhole limpet hemocyanin; MGG, isologous mouse gamma globulin; PFC, plaque-forming cells; T cells, thymus-derived lymphocytes; TATA, tumor-associated transplantation antigen; TNP, 2,4,6-trinitrophenyl.

cell functions in the induction of immune responses (6–8), and may provide a great advantage in analyzing T-T-cell interaction (9, 10).

The main advantages of the hapten-reactive T lymphocyte model are summarized as follows: (a) hapten-reactive T lymphocytes are capable of reacting with the haptens coupled on any antigenic molecule such as on the allogeneic and syngeneic tumor cells. Thus, one can easily analyze the regulatory role of T cells for the induction of cell-mediated immunity to such cell-associated antigens. (b) The helper and suppressor T-cell population can be raised against a simple hapten. One can analyze these antagonistic effects of T-cell populations on various immune responses (9). (c) Finally, as described before (7, 10), the hapten-reactive suppressor and helper T lymphocytes can be specifically inactivated by the treatment with hapten-D-GL conjugate (copolymer of D-glutamic acid and D-lysine). The ample difference in susceptibility to the hapten-D-GL conjugate between helper and suppressor T lymphocytes can be applicable to the system where one desires to modify arbitrarily one of these activities. This system may be especially relevant to the establishment of a model system for manipulation of various immune responses to the appropriate directions, i.e., suppression or enhancement.

In the present study, 2,4,6-trinitrophenyl (TNP)-residue was introduced to the surface of tumor cells as additional determinants, and hapten-reactive T lymphocyte activity was generated in mice by immunization with TNP-derivatized isologous mouse gamma globulin (MGG). Amplifying effect of TNP-reactive T cells on the tumor-specific killer T-cell-generation was analyzed by immunizing with TNP-derivatized tumor cells. The results will show the augmentation of induction of tumor-specific immunity by hapten-reactive T-cell activity, and provide evidence that appropriate manipulations designed to induce additional determinant-reactive helper (amplifier) T lymphocytes afford a potentially effective mode of immunoprophylaxis against the tumor.

## Materials and Methods

*Mice.* C3H/He and C57BL/6 mice of both sexes, originally obtained from The Jackson Laboratory (Bar Harbor, Maine) and maintained in our Institute, were used at 7–9 wk of age.

*Antigens and Preparation of Hapten-Carrier Conjugates.* Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem, San Diego, Calif., and human gamma globulin (HGG), Cohn fraction II was obtained from Nutritional Biochemical Corporation, Cleveland, Ohio. Mouse gamma globulin (MGG) was prepared from pooled serum of normal C3H/He and C57BL/6 mice by precipitation with 40% saturated ammonium sulfate and purified through diethyl-aminoethyl cellulose column chromatography with 0.015 M sodium phosphate buffer, pH 8.0. The D-GL was obtained from Pilot Chemicals, Inc., Watertown, Mass. The polymer had an average mol wt of 27,000 and a ratio of glutamic acid to lysine residues of 60:40.

The following TNP-conjugates were prepared by using sodium 2,4,6-trinitrobenzene sulfonate: $TNP_{32}$-HGG, $TNP_{27}$-MGG(C57BL/6), $TNP_{30}$-MGG(C3H/He), $TNP_{37}$-D-GL, and $TNP_{20}$-KLH. Subscripts refer to the average number of TNP groups per molecule of the proteins, and these were calculated from the absorption reading at 348 nm.

Benzylpenicilloyl (BPO)-KLH was prepared by reacting 100 mg of KLH with 100 mg of benzylpenicillin in 5 ml borate-buffered saline at pH 9.0 adjusted with 1 N NaOH. 14 molecules of BPO-group were found to bind covalently with 1 molecule of KLH as measured by the penamaldate method. $BPO_{14}$-MGG(C57BL/6)-$TNP_{27}$, $BPO_{15}$-MGG(C3H/He)-$TNP_{30}$, and $BPO_{14}$-KLH-$TNP_{20}$ were prepared from $TNP_{27}$-MGG(C57BL/6), $TNP_{30}$-MGG(C3H/He), and $TNP_{20}$-KLH by further reacting with benzylpenicillin as described above.

In the above calculations of numbers of haptens per molecule of carrier, the mol wt of KLH, MGG, and HGG were taken as 100,000, 160,000, and 160,000, respectively.

*Assay System for Measuring the Helper and Suppressor Activities of TNP-Reactive T Lymphocyte Populations.* Mice were immunized by intraperitoneal (i.p.) injection of 100 μg of syngeneic TNP-MGG in complete Freund's adjuvant (CFA). 3–8 wk after the immunization, single-cell suspensions were prepared from the spleens of these primed mice in Eagle's minimal essential medium (MEM), and used as the source of TNP-reactive T cells. Single-cell suspensions from spleens of mice which had been immunized i.p. 8–10 wk earlier with 100 μg of BPO-KLH in CFA were used as responding B cells, and transferred intravenously (i.v.) into 600 rads X-irradiated recipients together with TNP-MGG-primed cells. In other experiments, these TNP-

MGG-primed mice were directly X-irradiated and transferred with BPO-KLH-primed cells. For measuring the helper T-cell activity secondary antigenic challenges were performed by i.p. injection of 100 µg of BPO-MGG-TNP immediately after the cell transfer.

For measuring the suppressor T-cell activity, the above adoptively transferred recipient mice were i.p. challenged with BPO-KLH-TNP (9).

Helper and suppressor T-cell activities in the TNP-MGG-primed cell population were detected by the magnitude of anti-BPO antibody responses of transferred BPO-specific B cells as compared with those in the presence of normal cells. Anti-BPO antibody responses were measured by hemolytic plaques in recipient's spleens 7 days after adoptive transfer and antigenic stimulation.

*Hemolytic Plaque Assay.* Anti-BPO plaque-forming cells (PFC) in the spleens of recipient mice were counted by using a modification of Cunningham's hemolytic plaque technique as described previously (9). Since the number of direct PFC was always negligible, only indirect PFC are listed in the Results.

*Treatment for T-Cell Deprivation.* Mice were thymectomized at 6 wk and injected i.p. with 0.25 ml of anti-thymocyte serum (ATS) 5 d after thymectomy. ATS was prepared according to the two-pulse method introduced by Medawar and Levey (11), except that immunization was made with the thymocytes from C3H/He rather than from CBA mice. Efficiency of this procedure for T-cell deprivation was evaluated by determining both the susceptibility of spleen cells to anti-Thy-1 antiserum and guinea pig complement treatment and antibody response to thymus-independent antigen 5 days after the administration of ATS.

*Antibody Response to Thymus-Independent Antigen.* ε-Amino-dinitrophenyl-lysine-substituted dextran (DNP-dextran) was prepared by using a modification of the method of Fielder et al. (12), as described previously (13). After i.v. injection of 200 µg of DNP-dextran, direct anti-DNP PFC in the spleens were enumerated 5 d later. For the assay, sheep erythrocytes were conjugated with DNP-BSA by using $CrCl_3$ as described previously (9).

*Induction of Tumor-Specific Immunity.* As a tumor, spontaneously occurring X5563 plasmacytoma, derived from C3H/He strain and maintained by serial i.p. passages into syngeneic mice in ascitic form, was used. This tumor cell is free of surface immunoglobulin as detected by fluorescent antibody technique. $10^7$–3 × $10^7$ of TNP-derivatized tumor cells as described below were inoculated i.p. to induce specific immunity against tumor. For detecting protective immunity against tumor, $10^6$ of viable unmodified tumor cells were challenged intradermally (i.d.).

*Trinitrophenylation of Tumor Cells.* TNP-derivatized tumor cells were prepared according to the method described by Philpott et al. (14). Briefly, 100 × $10^6$ tumor cells pretreated with mitomycin C at a concentration of 40 µg/ml for 50 min at 37°C were suspended in 100 ml of Hanks' balanced salt solution containing 300 µg/ml of sodium TNP sulphonate. After 30 min incubation at 37°C, the tumor cells were washed twice and viability of the cells was determined by trypan blue dye exclusion test. By this system, more than 90% of the tumor cells were viable after coupling with TNP.

*Assay System for Killer T-Cell Activity.* Cytotoxicity assay, originally devised by Hashimoto and Sudo (15) was utilized with a slight modification into microculture-plate system. Radiolabeling of tumor cells was performed by incubation rocking of a suspension of 3 × $10^6$ of tumor cells in 3 ml of RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (RPMI-FCS) and 1 µCi of [$^3$H]uridine (the Radiochemical Centre, Amersham, England) (sp act 2.9 Ci/mmol) in a Falcon 3002 tissue culture dish, at 37°C for 60 min. After extensive washings, 3 × $10^4$ of radiolabeled target tumor cells suspended in 0.1 ml of RPMI-FCS were distributed into each well of a Falcon 3040 microplate, and mixed together with the graded numbers (75–300 × $10^4$) of normal or immunized spleen cells suspended in 0.1 ml of RPMI-FCS. After effector to target interaction was proceeded for 6 h at 37°C in 5% $CO_2$/air, the cells in each well were harvested and washed twice with a large volume of cold 5% trichloroacetic acid solution to eliminate all the radioactivity associated with killed target cells, and the radioactivity remaining in the viable target cells was measured in a liquid scintillation counter. Cytotoxicity index (percentage specific killing) was calculated as: percentage specific killing = (1 − [cpm in the immune well/cpm in the normal well]) × 100%, where the normal or immune well denotes target cells incubated with normal or immune spleen cells. In the above cytotoxicity test, the values listed in the Results represent the mean of four immune wells and

the final target cell viability in the normal wells was usually in the range of 95–100% of that at the initiation of the assay.

In our previous study (16), it was established that the cytotoxic activity detected in X5563-C3H/He combination was mediated exclusively by killer T lymphocytes but not by antibody-dependent K-cell activities or cytotoxic macrophages.

*Measurement of Tumor Growth.* Tumor growth was measured by quantitation of myeloma protein in sera of tumor-inoculated mice with radial immunodiffusion technique utilizing rabbit anti-idiotypic antibody (17).

## Results

*Generation of TNP-Reactive Helper T-Cell Activity by TNP-MGG-Priming.* Previously, it was demonstrated that hapten-reactive helper T cells could be raised in mice by immunization with hapten-derivatized isologous MGG (6–8). These helper T cells collaborate with another hapten-specific B lymphocyte at the stimulation with isologous MGG derivatized with double haptens of distinct specificities. Consistent with the previous observations, as shown in Table I, TNP-reactive helper T-cell activity was raised in C57BL/6 mice by immunization with TNP-MGG and the activity was detected by transferring BPO-specific B cells into 600 rads X-irradiated TNP-MGG-primed mice and stimulating with BPO-MGG-TNP (group II).

The failure to detect such helper activity in MGG-primed mice suggests the TNP-specificity of helpers (group III). The helper activity in TNP-MGG-primed mice was not detected by the stimulation with BPO-MGG (not conjugated with TNP), further indicating the TNP-reactivity of such helper cells (data not listed). Moreover, the failure to detect such TNP-reactive helper cell activity in TNP-HGG-primed recipients (group IV) is reminiscent of the previous observation (7) that hapten-reactive helper T-cell activity could not be raised in mice by the immunization with hapten-heterologous protein conjugates. This also indicates that TNP-primed B cells or circulating anti-TNP-antibodies are not responsible for these helper cell activities.

In this experiment, because the helper cell activity was detected in heavily irradiated TNP-MGG-primed mice, it can be also concluded that TNP-reactive helper cell activity is radioresistant.

*Amplifying Effect of TNP-MGG-Priming on the Generation of Cytotoxic T Lymphocytes to Alloantigen at the Immunization with TNP-Modified Allogeneic Tumor Cells.* To test the

TABLE I

*Generation of TNP-Reactive Helper T-Cell Activity by Immunization with TNP-MGG\**

| Exp. group | C57BL/6 mice primed with | Responding B cells transferred | 2nd Ag | Anti-BPO antibody response (PFC/spleen)‡ |
|---|---|---|---|---|
| I | None | | | 335 (1.43) |
| II | TNP-MGG | C57BL/6 | BPO-MGG-TNP | 4,062 (1.06) |
| III | MGG | BPO-KLH- | | 886 (1.46) |
| IV | TNP-HGG | Primed cells | | 518 (1.47) |

\* C57BL/6 mice were primed by i.p. injection of either 100 μg of TNP-MGG (C57BL/6), MGG (C57BL/6), or TNP-HGG in CFA 6 wk before cell transfer. Those primed mice and other group of nonimmunized normal mice were X-irradiated (600 rads) and then injected i.v. with 50 × 10⁶ of BPO-KLH-primed spleen cells. All recipients were stimulated i.p. with 100 μg of BPO-MGG-TNP. The BPO-KLH-primed spleen cells came from syngeneic donors which had been immunized with 100 μg of BPO-KLH in CFA 8 wk previously.

‡ The values denote geometric means and standard errors in the recipient spleens 7 days after the cell transfer. Numbers in parentheses represent standard errors.

TABLE II

*Amplifying Effect of TNP-MGG-Priming on the Generation of Cytotoxic T Lymphocytes to Alloantigen at the Immunization with TNP-Modified Allogeneic Tumor Cells\**

| Exp. group | C57BL/6 mice treated with | | Killer T-cell activity | |
|---|---|---|---|---|
| | Hapten-priming | Tumor-immuniza-tion | X5563 | TNP-X5563‡ |
| | | | | % |
| I | CFA | TNP-X5563 | 0 | 0 |
| II | TNP-MGG | TNP-X5563 | 40 | 50 |
| III | MGG | TNP-X5563 | 0 | 3 |
| IV | TNP-HGG | TNP-X5563 | 2 | 2 |
| V | TNP-MGG | None | 3 | 6 |

\* C57BL/6 mice were preimmunized with TNP-conjugates as indicated, and 6 wk later $10^7$ of TNP-derivatized X5563 cells (C3H/He origin) were i.p. injected. 1 wk after the tumor-immunization, cytotoxic killer T-cell activities of spleen cells were measured against X5563 and TNP-X5563 at target to effector cell ratio of 1:50.

‡ The values represent percent specific killing to respective targets.

effect of TNP-MGG-priming on the generation of alloantigen-reactive killer T lymphocytes subsequent to the immunization with TNP-derivatized allogeneic tumor cells, $10^7$ of TNP-derivatized X5563 (C3H/He origin) were injected i.p. into C57BL/6 mice 6 wk after the TNP-MGG, MGG, or TNP-HGG-priming. As shown in Table II, killer T cells to the allogeneic tumor cells were not induced in CFA-primed control animals by the immunization with TNP-X5563 in this experimental condition (group I). On the other hand, TNP-MGG-preimmunization apparently augmented the generation of killer T cells to the allogeneic X5563 tumor cells (group II). Other preimmunization regimens such as of MGG or TNP-HGG revealed no significant enhancing effects (groups III and IV), indicating that the preimmunization regimen which generates the TNP-reactive helper T-cell activity as in Table I was a prerequisite for this augmentation. The specificity of these cytotoxic killer T cells is directed mainly to alloantigens on X5563 and was verified by the comparable cytotoxicity to unmodified X5563 and TNP-modified X5563 cells.

*Radioresistant Nature of Amplifying Activity in TNP-MGG-Primed Mice on the Generation of Killer T Lymphocytes.* It was not certain whether TNP-reactive amplifying activity in the TNP-MGG-primed mice was equivalent to the TNP-reactive helper activity for the induction of antibody responses which had been shown in Table I. However, it was revealed that TNP-reactive amplifier activity had the same functional property as helper activity with respect to its radiation resistance. C57BL/6 mice were immunized with TNP-MGG in CFA or only CFA as control. 6 wk later, half of the mice in each group was 600 rads X-irradiated and reconstituted with $10^8$ of normal spleen cells as the responder killer cell source, and the other half was nonirradiated. The irradiated and nonirradiated mice were then immunized two times every week with 2 × $10^7$ of TNP-conjugated X5563. 7 d after the last immunization, killer T cell activities against allogeneic X5563 tumor cells were tested at a target to effector ratio of 1:50.

As shown in Table III, it is noticeable that the comparable augmentation by TNP-MGG-preimmunization was elicited irrespective of whether or not these TNP-MGG-

TABLE III

*Radioresistant Nature of Amplifying Activity in TNP-MGG-Primed Mice on the Generation of Killer T Lymphocytes**

| C57BL/6 Mice treated with | | | Killer T-cell activity X5563 |
| Hapten-priming | X-irradiation | Hapten-derivatized tumor-immuniza-tion | |
| --- | --- | --- | --- |
| | | | % |
| CFA⎫<br>TNP-MGG⎭ | None | TNP-X5563<br>TNP-X5563 | 25.2<br>48.0 |
| CFA⎫<br>TNP-MGG⎭ | 600 rads X-irradiated‡ and reconstituted with normal spleen cells | TNP-X5563<br>TNP-X5563 | 31.2<br>57.2 |

* C57BL/6 mice were preimmunized with 100 μg of TNP-MGG (C57BL/6) in CFA or CFA alone as control 6 wk previously. 600 rads X-irradiation of those primed animals and reconstitution with $10^8$ of normal spleen cells were performed immediately before the TNP-tumor immunization. Killer T-cell activities generated in those animals against allogeneic X5563 cells were tested at target to effector ratio of 1:50, 7 days after the two consecutive weekly immunizations with 2 × $10^7$ TNP-X5563.

‡ In a separate experiment, proliferating activity of lymphocytes in host was completely lost by this dose of X-irradiation as assessed by mixed lymphocyte reactivity to alloantigens.

primed mice received whole body X-irradiation. Thus, the amplifying effect of TNP-MGG-preimmunization is radioresistant, and functionally similar to the helper cell activity (18).

*Requirement of T-Cell Population for Amplifying Activity in TNP-MGG-Primed Mice.*    To ascertain further T lymphocytes in TNP-MGG-primed mice mediating this amplified generation of killer T cells, T-cell-deprived C57BL/6 mice were prepared with adult thymectomy (ATX) plus anti-thymocyte serum (ATS)-treatment, and primed with TNP-MGG as above. The efficiency of the methods used here for T-cell depletion was determined by measuring the quantity of Thy-1-positive cells remaining in the spleen 5 d after T-cell depletion. As shown in Table IV, the number of Thy-1-positive cells decreased in (ATX + ATS)-treated mice, whereas B-cell activity was not affected, as evident from the direct anti-DNP antibody responses to the thymus-independent antigen, DNP-dextran.

Normal or those T-cell-deprived mice were primed with TNP-MGG, and 6 wk later, 600 rads X-irradiated and reconstituted i.v. with $10^8$ of normal spleen cells as the responder killer T-cell source. TNP-X5563 tumor cell-immunization was started immediately after the cell transfer, and boosted 1 wk later. As the results are shown in Table IV, the amplifying effect of TNP-MGG-priming on the generation of killer T cells as demonstrated in normal animals (group II) was not observed in (ATX + ATS)-treated group (group III). It can be concluded, therefore, that the T-cell population in TNP-MGG-primed mice plays a crucial role in the amplification of generation of killer T-cell activity in the above allogeneic host-tumor combination.

*Marginal Augmentation of Killer T-Cell Development against TATA by the Immunization with TNP-Modified Syngeneic Tumor Cells to TNP-MGG-Preimmunized Mice.*    While the TNP-MGG-primed allogeneic C57BL/6 mice clearly augmented the generation of cytotoxic T cells to the C3H/He plasmacytoma by immunization with TNP-modifi-

TABLE IV

*Failure to Detect the Amplifying Effect of TNP-MGG-Priming on the Generation of Cytotoxic T Lymphocytes in T Cell-Deprived Mice*

| Exp. group | C57BL/6 Mice | Susceptibility of spleen cells to anti-Thy-1 anti-sera and complement* Killing | B-cell response against DNP-dextran‡ Anti-DNP PFC/spleen | TNP-MGG-priming§ | TNP-X5563 immunization | Killer T-cell activity X5563 |
|---|---|---|---|---|---|---|
| | | % | | | | % |
| I | Normal | — | — | No | Yes | 18.5 |
| II | Normal | 26.3 (100) | 42,300 (1.16) | Yes | Yes | 46.5 |
| III | ATX + ATS ‖ | 1.8 (6.8) | 40,780 (1.25) | Yes | Yes | 21.2 |

* Thy-1 positivity was determined in the spleen 5 d after the injection of ATS. The values represent the means of three mice.

‡ Normal or T-cell-deprived mice were immunized i.v. with 200 μg of thymus-independent antigen, DNP-dextran, 5 d after the injection of ATS. After 5 d, direct anti-DNP PFC in the spleen were measured and expressed as the geometric means of four animals.

§ Normal or T-cell-deprived mice were 600 rads X-irradiated and reconstituted with $10^8$ of normal spleen cells, followed by the priming with TNP-MGG. 6 wk later, all mice were immunized i.p. with $10^7$ of TNP-X5563, and boosted 7 d later. Killer T-cell activity was measured 7 d after the last immunization at target to effector ratio of 1:50.

‖ C57BL/6 mice were thymectomized at 6 wk and injected i.p. with 0.25 ml of ATS 5 d after thymectomy.

TABLE V

*Marginal Augmentation of Killer T-Cell Generation against X5563 TATA by Hapten-TATA Cooperation*

| C3H/He Mice treated with | | Killer T-cell activity | |
|---|---|---|---|
| Hapten-priming | Hapten-derivatized tumor-immunization | X5563 | TNP-X5563 |
| | | % | |
| CFA | TNP-X5563 | 9 | 8 |
| TNP-MGG | TNP-X5563 | 15 | 23 |

* C3H/He mice which had been preimmunized with 100 μg of TNP-MGG (C3H/He) in CFA or CFA alone 8 wk previously were immunized with 3 × $10^7$ of TNP-X5563 (syngeneic tumor) four times i.p. at 2-wk intervals. Cytotoxicity assay was performed 2 wk after the last immunization at target to effector cell ratio of 1:100.

cation, it was investigated whether syngeneic C3H/He mice can also respond to the same TNP-derivatized tumor cells with this TNP-MGG-preimmunization. As one of the typical results is shown in Table V, the preimmunization with TNP-MGG only slightly augmented the generation of killer T cells against TATA at repeated immunization with TNP-modified tumor cells. However, such amplifying effect of TNP-MGG-preimmunization on the killer T lymphocytes in syngeneic combination was not consistently reproduced in all the experiments. It was assumed that coexistence of TNP-reactive suppressor T-cell activities in those TNP-MGG immunized mice may diminish or interfere with the TNP-reactive helper (amplifier) T-cell activities as described in the previous report (10). In the following section, a trial was made to eliminate the suppressor T-cell activity from TNP-reactive T-lymphocyte populations.

*Selective Inhibition of Generation of TNP-Reactive Suppressor T-Cell Activity by TNP-D-GL-*

Table VI

*Selective Inhibition of Generation of TNP-Reactive Suppressor T-Cell Activity by TNP-D-GL-Pretreatment and Resulting Augmented Generation of TNP-Reactive Helper T-Cell Activity*[*]

| Responding cells | Exp. group | Condition for generation of TNP-reactive T cell in C3H/He Mice | | 2nd Ag (activity detected) | Anti-BPO antibody response | |
|---|---|---|---|---|---|---|
| | | TNP-D-GL pretreatment | Hapten-priming | | PFC/spleen | Index |
| BPO-KLH-primed cells | I | None | CFA ⎫ | | 1,656 (1.22) | 1.00 |
| | II | None | TNP-MGG ⎬ | BPO-MGG-TNP | 3,084 (1.26) | 1.86 |
| | III | 500 μg TNP-D-GL | TNP-MGG ⎭ | (Helper) | 8,577 (1.01) | 5.18 |
| | IV | None | CFA ⎫ | | 58,504 (1.09) | 1.00 |
| | V | None | TNP-MGG ⎬ | BPO-KLH-TNP | 21,926 (1.17) | 0.37 |
| | VI | 500 μg TNP-D-GL | TNP-MGG ⎭ | (Suppressor) | 51,423 (1.24) | 0.88 |

[*] C3H/He mice were either pretreated with TNP-D-GL 3 days before TNP-MGG (C3H/He)-immunization or not pretreated. The TNP-reactive helper and suppressor T-cell activities generated in these TNP-MGG-primed mice were measured 3 wk after the TNP-MGG-immunization by transferring their spleen cells into other 600 rads X-irradiated recipient mice together with BPO-KLH-primed cells and stimulating with 100 μg of BPO-MGG-TNP or BPO-KLH-TNP. The BPO-KLH-primed cells came from another donor mice which had been immunized 10 wk previously.

*Pretreatment and Resulting Augmented Generation of TNP-Reactive Helper T-Cell Activity in TNP-MGG-Preimmunized Mice.* Previously (10) it was found that hapten-reactive suppressor T-cell activity was more sensitive to the inactivation by hapten-conjugated nonimmunogenic copolymer, D-GL, than helper T cells. Moreover, the inhibition of suppressor T-cell generation by the pretreatment with hapten-conjugated D-GL before the immunization of hapten-isologous protein conjugate resulted in an augmentation of the development of hapten-reactive helper T-cell activity. Consistent with the previous observations, as shown in Table VI, TNP-reactive helper T-cell activity was clearly augmented by the treatment of TNP-D-GL before TNP-MGG immunization (comparison between groups II and III). The notion that augmentation of TNP-reactive helper T-cell activity was due to the inhibition of generation of TNP-reactive suppressor T cells by TNP-D-GL-treatment, was substantiated by the comparison between groups V and VI. As shown in group V, anti-BPO antibody responses were substantially lower than group IV (control), and this reduction as reflected by the TNP-reactive suppressor T-cell activity in TNP-MGG-primed cell population was not observed in the cell population pretreated with TNP-D-GL before TNP-MGG-priming (group VI).

Although the data are not shown here, that the relevant cells in TNP-MGG-primed cell population mediating the TNP-reactive helper and suppressor functions are T cells, and not B cells, was indicated by the following two experimental results. First, TNP-MGG-primed spleen cells were depleted of B cells by treatment with an antiserum specific for antigens on the surface of mouse B cells, known as anti-Th-B (19). The almost complete reduction of B-cell activity in the anti-Th-B treated TNP-MGG-primed spleen cells contrasts sharply with the intact helper and suppressor cell activities. Second, the TNP-MGG-primed spleen cells were treated with either normal mouse serum plus C or anti-Thy-1,2 antiserum plus C. Both helper and suppressor cell activities were almost completely abolished by treatment with anti-Thy-1,2 antiserum plus C. These results were exactly in corroboration with those in para-azobenzoate reactive T cells as demonstrated previously (9). Thus, helper and suppressor cell activities developed by immunization with TNP-MGG were both mediated by Thy-1-positive T lymphocytes.

Table VII

*Amplifying Effect of TNP-D-GL-Pretreatment on the Generation of Killer T Cells to Syngeneic Tumor Cells in the Process of Hapten-TATA T-T Cell Interactions*\*

| Exp. group | Conditions for generation of TNP-reactive T cells in C3H/He mice | | Hapten-derivatized tumor-immuniza-tion | Killer T-cell activity X5563 |
|---|---|---|---|---|
| | TNP-D-GL-pretreatment | Hapten-priming | | |
| | | | | % |
| I | None | CFA | TNP-X5563 | 0.5 |
| II | None | TNP-MGG | TNP-X5563 | 8.4 |
| III | 500 μg TNP-D-GL | TNP-MGG | TNP-X5563 | 47.1 |

\* C3H/He mice either pretreated with TNP-D-GL 3 d earlier or not pretreated were immunized with 100 μg of TNP-MGG (C3H/He) in CFA. Sham-immunized mice (group I) received CFA alone. 6 wk thereafter, those mice were immunized i.p. with $10^7$ of TNP-derivatized syngeneic tumor cells once a week for 5 wk, and killer T lymphocyte activities generated in the spleens were measured 7 d after the last immunization at target to effector cell ratio of 1:100.

In conclusion, the selective elimination of suppressor T cells by TNP-D-GL-treatment resulted in an augmented helper T-cell activity.

*Amplification of Generation of Tumor-Specific Killer T Lymphocyte Activity by Suppressor T-Cell-Depleted Hapten-Reactive T Lymphocytes.* Having established a reproducible system for a potent induction of TNP-reactive helper T lymphocytes in C3H/He mice, the feasibility of utilizing this helper T-cell system to facilitate development of tumor-specific immunity was explored. The TNP-derivatized X5563 as above was used as an immunizing antigen and the capacity of recipient mice to develop tumor-specific cytotoxic T cells was tested depending on whether or not such mice had been preimmunized in a manner to develop potent TNP-reactive helper T-cell activities. The results are summarized in Table VII. C3H/He mice preimmunized with TNP-MGG 6 wk previously and repeatedly immunized with TNP-conjugated tumor cells (group II) developed specific cytotoxic activity only marginally higher than that observed in sham (CFA)-preimmunized control mice (group I). In contrast, the mice which had been immunized with TNP-MGG in conjunction with TNP-D-GL in such a manner as to develop more potent TNP-reactive helper T-cell activities (group III), generated a striking magnitude of killer T-cell activity.

*Augmenting Effect of TNP-D-GL-Pretreatment in TNP-MGG-Primed Mice on the Development of Tumor-Specific Immune Resistance.* The obvious aim of this type of approach was to develop an effective method for substantially improving host anti-tumor immunity against growth of syngeneic tumors. The following results indicate that it is possible to improve anti-tumor immunity by this method as reflected by a significant prolongation of host survival after inoculation of lethal viable tumor cells. Thus, the experimental protocol followed substantially the same as Table VII, except that the mice were challenged i.d. with lethal doses ($10^6$) of X5563 cells 7 days after the last TNP-derivatized tumor-immunization. These results are summarized in Fig. 1. Thus, sham (CFA)-immunized (group I) as well as TNP-MGG-immunized C3H/He mice without TNP-D-GL-pretreatment (group II) failed to develop any appreciable tumor-specific immune-capability after immunization with TNP-derivatized X5563 syngeneic tumor cells. On the other hand, the mice (group III), which had generated potent TNP-reactive helper T-cell activity with TNP-D-GL pretreatment before TNP-MGG-priming displayed higher levels of immune resistance against the challenge with
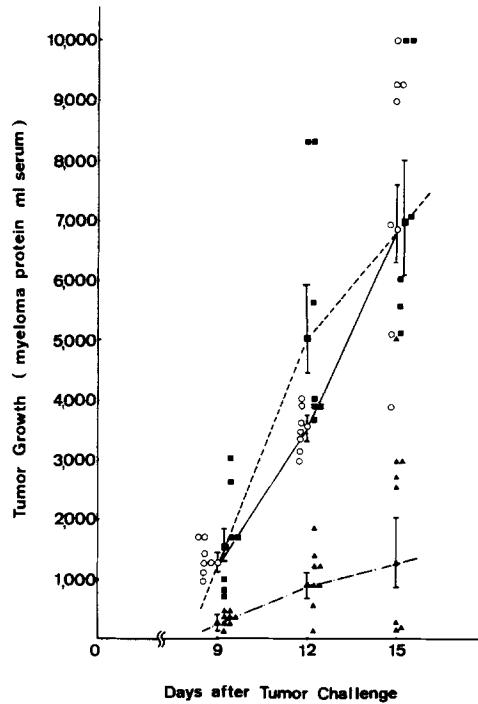
**Days after Tumor Challenge**

Fig. 1. Augmenting effect of TNP-D-GL-pretreatment on the development of tumor-specific immunity in hapten-TATA T-T-cell interactions. Experimental protocol was substantially the same as Table VII, except that the mice were challenged i.d. with lethal dose ($10^6$) of viable syngeneic X5563 tumor cells 7 days after the last TNP-derivatized tumor-immunization. Tumor-growth was quantitatively determined by the concentration of myeloma protein in serum, and this was measured by utilizing anti-idiotypic antibody to X5563 myeloma protein. Group I (O), group II (■), and group III (▲), received exactly the same pretreatments and immunizing regimens as in Table VII, respectively. Individual values of tumor growth in each group, and geometric means and standard errors are illustrated. The ultimate tumor-death values in groups I, II, and III were 7/7, 7/7, and 5/8, and mean survival times (days) of dead animals in those groups were 17.8 ± 0.70, 16.3 ± 1.02, and 25.1 ± 1.31, respectively.

viable tumor, and the growth of challenged tumor was significantly suppressed. Moreover, as shown by mortality values of the respective groups summarized in the legend of Fig. 1, mice subjected to this preimmunization regimen are also appreciably improved in terms of their capacity to resist tumor growth after inoculation of a lethal dose of such viable tumor cells, and in the significant number of animals (three out of eight) the challenged tumor regressed. Thus, the above results clearly indicate that appropriate manipulations designed to induce the potent hapten-reactive helper T-lymphocyte activity provide a very effective mode of immunopotentiation against tumor.

## Discussion

The use of chemically modified tumor cells to be treated for specific, active immunization has been receiving considerable attention. The attractiveness of this approach stems from its potential for both therapy and prophylaxis. The theoretical framework for the role of cell co-operation in tumor immunity, and speculations on manipulations that might augment tumor rejection have been presented by Mitchison

(20). His work showed that the immunogenicity of tumor antigens might be augmented by coupling new helper antigens such as haptens, proteins, new transplantation antigens, viral coat antigens, or xenogeneic cell antigens to tumor cell surfaces. In the past decade a variety of experimental observations suggested that such a mechanism may indeed result in increased resistance to in vivo tumor growth. Martin et al. (21) were able to detect a slight but significant increased cell-mediated cytotoxicity for native tumor cells in the spleens of C57BL/6 mice immunized with concanavalin A-or dinitrophenylaminocaproate-coated EL-4 cells. Hashimoto and Yamanoha (22) also reported that C57BL/6 mice immunized with EL-4 cells coupled with fluorescent dansyl group in aqueous medium by dansyl chloride-cyclohepta amylose complex aquired transplantation immunity specific for the tumor cells. Likewise, Galili et al. (23) demonstrated that trinitrophenylated Moloney virus-induced YAC cells induced a higher cytotoxic antibody response and better protection against small tumor cell doses in syngeneic, low responsive strain A mice than nonmodified YAC cells that had been inactivated by irradiation or mitomycin C treatment. These results indicated that low responsiveness of syngeneic hosts to the tumor antigens can be overcome, at least to some extent, by coupling the immunizing tumor cells to a strong immunogenic hapten, yet we believe that the mechanisms remain obscure, and the augmenting activity is not consistently high enough.

In the present study, we studied the above notion more directly by using hapten-TATA T-T-cell collaboration system for the induction of tumor-specific transplantation immunity. TNP-residue was introduced on the surface of tumor cells as additional determinants because haptenation with TNP could be achieved with minimum denaturation of cell-surface component and TNP-reactive helper T lymphocyte activity was easily generated in mice by immunization with TNP-derivatized MGG. Thus, the amplifying effect of TNP-reactive helper T cells on the TATA-reactive killer T-cell-generation could be easily analyzed by immunizing with TNP-derivatized tumor cells.

In a combination of C57BL/6 mice and TNP-modified X5563 allogeneic tumor, TNP-MGG-preimmunization which successfully generated the TNP-reactive helper T-cell activity (Table I) augmented the generation of killer T cells to the allogeneic X5563 tumor cells (Table II). An allogeneic model system was used to study the optimal conditions for the chemical modification procedure of the tumor cells and appropriate immunization regimens. Based on the positive augmenting effect of TNP-MGG-priming, the same principle was applied to the syngeneic combination. The result (Table V) demonstrated that the preimmunization with TNP-MGG only slightly augmented the generation of killer T cells against TATA at repeated immunization with the same TNP-modified X5563 cells. However, such amplifying effect of TNP-MGG-preimmunization on the killer T-cell generation in syngeneic combination was rather weak, and not always reproduced in all the experiment. In the allogeneic combination, it has been established that amplifier T cells directed against lymphocyte-defined determinants of H-2 complex collaborate with killer T cells directed against serologically-defined determinant (24). When a new antigenic determinant (TNP) was introduced on the surface of allogeneic cells and immunized in the presence of TNP-reactive helper T cells, it is conceivable that the TNP-reactive T cells collaborate with such amplifier T cells responsible for generation of alloantigen-reactive killer T cells besides the collaboration with killer T cells, and resulted in an augmentation of the generation of killer T-cell activity. On the other hand, in the

syngeneic system, the killer T cells are only the relevant cell type reactive with TATA of X5563 as mentioned earlier (3), and TNP-reactive T cells may solely function as amplifier T cells for the generation of killer T cells. This possible mechanistic distinction may explain the difference in augmenting effect of TNP-reactive T cells on the generation of killer T lymphocytes between allogeneic and syngeneic combinations.

The other possible reason for the weak augmenting effect of sole TNP-MGG-priming on the generation of TATA-specific killer T lymphocytes in syngeneic combination, is that the coexistence of TNP-reactive suppressor T-cell activities generated in the TNP-MGG-preimmunized mice may diminish or interfere with the potential activities of TNP-reactive helper T-cell population. Previously (10), the influence of inhibiting suppressor T-cell generation on the development of helper T cells was analyzed with respect to hapten-specificity of the latter's responsiveness. The functional specificity of helper T-cell activity could be quantitatively measured by the responses of a hapten-reactive T-cell population to various haptenic determinants of structurally-related compounds. The conclusion that suppressor T cells generated during the primary immune response ultimately exert an effect on helper T-cell development derived from the following observations: (a) that the development of helper T-cell activity was substantially augmented in the hapten-D-GL-pretreated animals in which the generation of suppressor T-cell activity was completely inhibited; and (b) that the hapten-reactive T lymphocyte populations developed in the absence of suppressor T cells have a unique quality of their higher specificity and affinity for haptenic determinants to which they have been primed.

Consistent with the previous observations (10), in the present study the ability of TNP-D-GL-treatment to selectively inactivate TNP-reactive suppressor T-cell activity was also demonstrated by the type of experiment summarized in Table VI, and resulted in a striking increase in helper T-cell activity. Corroboration with this augmented helper T-cell generation, the immunization with TNP-modified syngeneic tumor cells into such animals resulted in the striking levels of generation of specific cytotoxic T-cell activity (Table VII), and the corollary protective effect of such treatment as is illustrated by the significant prolongation of survival in animals after inoculation of a lethal dose of viable tumor cells (Fig. 1). These results clearly indicate, therefore, that appropriate manipulations designed to induce potent hapten-reactive helper T lymphocyte activity can be established to provide a potentially very effective mode of immunoprophylaxis against syngeneic tumor cells.

Although it is not yet certain from the present study whether or not the TNP-reactive amplifier T cells responsible for augmentation of tumor-specific immunity are equivalent to the helper T cells for triggering B lymphocytes, those T-cell activities are functionally indentical at least with respect to the following two properties: both activities are (a) X-irradiation-resistant, and (b) resistant to TNP-D-GL-treatment. The precise characterization of such amplifier T cells is under progress in our laboratory.

Finally, it may be of value to mention that the obvious goal of the application of hapten-reactive T-cell activity to the tumor system is to develop an effective mode of immunotherapy to substantially improve host anti-tumor immunity against already growing tumors. For example, if one wishes to induce effective host resistance against already growing tumor in this system, one approach would be to attempt to preimmunize an individual with hapten-conjugate autologous protein in conjunction with

appropriate pretreatment with the relevant hapten-D-GL copolymer, followed at some later time by immunization of the individual with hapten-coupled attenuated tumor cells. As demonstrated in the present experiments, it would be reasonable to assume that the hapten-reactive T cells induced by previous immunization in this manner facilitated the development of tumor-specific cytotoxic T lymphocytes and resulted in tumor rejection. However, further experiments to demonstrate the above hapten-reactive helper T-cell system to suppress the already growing tumor have been unsuccessful. The development of tumor-specific cytotoxic T lymphocytes was significantly depressed in the X5563 tumor-bearing animals, and the mechanism for this depression was ascribed to the presence of large quantities of tumor antigen derived from the tumor mass. Experimentally, this was induced by the repeated pretreatment with MMC-attenuated tumor cells (not conjugated with hapten), and this treatment significantly inhibited the development of tumor-immunity even after appropriate tumor immunization procedures. Thus, the pre or coexistence of inappropriate form of TATA inhibits the subsequent induction of anti-tumor immunity, and this suppressive mechanism is operating in the specific immunosuppression of tumor-bearing animals (16).

Based on the above consideration, we explored the feasibility of utilizing the hapten-reactive helper T lymphocyte system to the prevention of this type of suppression. If one can prevent the killer T-cell suppression induced by the presence of inappropriate form of TATA with the hapten-reactive helper T-cell activities, this approach may provide some premise for circumvention of tumor-specific immunodepression frequently observed in tumor-bearing animals. The results[2] revealed that the supplement of hapten-reactive T cells to the system at the pretreatment with MMC-attenuated tumor cells with haptenation invariably inhibited this type of suppression for induction of killer T lymphocytes, and turn the negative signal of this pretreatment to positive stimulus as demonstrated here. Thus, it is logical to conclude that approaches leading to direct amplification of effector activity on one hand, and to depletion of suppressive effects on the other, by hapten-reactive T-lymphocyte activity, may be one of the most direct routes to augment overall anti-tumor responses and for the immunotherapy of cancer.

## Summary

2,4,6-trinitrophenyl (TNP)-reactive T-cell activities were raised in mice by immunization with TNP-isologous mouse gamma globulin. After establishing that TNP-reactive T lymphocytes can serve as amplifier cells for induction of killer T lymphocytes in allogeneic system, we explored the possibility of this hapten-reactive T-cell system to amplify tumor-specific killer T-lymphocyte activity in the syngeneic system. We utilized relatively weak immunogenic syngeneic plasmacytoma X5563 in C3H/He mice.

Analysis of the TNP-reactive T-cell activities revealed that such T lymphocytes express the biological functions of both major subtypes of regulatory T cells, namely suppressors and helpers, and that TNP-reactive suppressor and helper T lymphocytes, respectively, differ in their relative susceptibility to specific inactivation by TNP

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[2] T. Hamaoka et al. Regulatory functions of hapten-reactive helper and suppressor T lymphocytes. IV. Prevention of tolerance induction in killer T cell lineage against tumor-specific transplantation antigen by supplement of hapten-reactive helper T cell activities. Manuscript in preparation.

conjugates of the nonimmunogenic D-amino acid copolymer, D-glutamic acid, and D-lysine (D-GL). By taking advantage of the relative susceptibility-difference to TNP-D-GL, selective inactivation of TNP-reactive suppressor T cells was induced by appropriate treatment with TNP-D-GL, and the generation of TNP-reactive helper T-cell activity was amplified.

The supplement of augmented TNP-reactive helper T-cell activity to the system at the immunization with syngeneic X5563 with TNP-haptenation, resulted in a striking augmentation of induction of tumor-specific killer T-lymphocyte activity, and a considerable number of hosts survived after the challenge with lethal dose of viable tumor cells.

Thus, appropriate manipulations designed to induce potent hapten-reactive helper T lymphocytes provided the potential for a very effective mode of immunoprophylaxis against tumor.

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