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Preferential generation of killer or helper T-lymphocyte activity directed to the tumour-associated transplantation antigens

H. FUJIWARA, T. HAMAOKA, KUMIKO TESHIMA, H. AOKI & M. KITAGAWA
Institute for Cancer Research, Osaka University Medical School, Fukushima, Fukushimaku, Osaka, Japan

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Summary. Induction of killer and helper T-cell activities towards transplantation antigens of two tumour cell lines was analysed in the allogeneic and syngeneic host combinations. The lymphoid cells from C57BL/6 mice immunized with allogeneic viable or mitomycin C-treated X-5563 plasmacytoma cells derived from C3H/He mice revealed both killer and helper T-cell activities against alloantigens, whereas cells from mice immunized with tumour cells killed by a freezing and thawing procedure revealed predominantly helper T-cell activity. On the other hand, when C3H/He mice were immunized with viable syngeneic X-5563 plasmacytoma or MM102 mammary tumour cells, the former generated preferentially killer T-cell activity, whereas the latter induced predominantly helper T-cell activity against tumour-associated transplantation antigens.

Thus, immunization with transplantation antigen(s) does not always induce both helper and killer T-cell activities in parallel, but a certain antigenic system induces predominantly one type of T-cell response, thus indicating that two distinct subsets of helper and killer T cells against the transplantation antigen(s) can be raised independently without an absolute requirement of collaboration between these different T-cell subsets.

INTRODUCTION

It has been well established that thymus-derived (T) lymphocytes mediate various immunological functions such as helper T cells in the induction of humoral immunity (Katz and Benacerraf, 1972), and killer T cells in cell-mediated immunity (Cerottini and Brunner, 1974; Wagner, Röllinghoff and Nossal, 1973). With respect to the interrelationship between these functionally heterogenous T-cell populations, it was recently demonstrated that helper T cells to sheep erythrocytes and killer T cells to allogeneic cells are generated from two distinct precursors with different Ly antigens (Cantor and Boyse, 1975a), and therefore belong to the mutually different subsets already committed for differentiation into respective cell types before the antigenic stimulation. Moreover, the amplifier T cells which are able to augment the generation of cytotoxic killer T cells against the major histocompatibility antigens, also have the same Ly specificity as helper T cells involved in antibody formation (Cantor and Boyse, 1975b). These observations provided us with very convincing evidence of the interaction of two distinct subsets of T cells responsible for the generation of killer T cells.

Dennert and Lennox (1974) and Igarashi, Okada, Kishimoto and Yamamura (1975) also reported independently that the immunization with viable allogeneic tumour cells was capable of generating predominantly cytotoxic T cells, whereas the immunization with allogeneic tumour cells killed by formaldehyde treatment or freezing and thawing procedure resulted in a generation predominantly composed of helper T cells, suggesting that preferential generation of helper T cells or killer T cells

Correspondence: Dr. H. Fujiwara, Institute for Cancer Research, Osaka University Medical School, Fukushima, Fukishimaku, Osaka, Japan.

to alloantigens can be accomplished with different immunization regimens. If these observations are consistent with all the alloantigen system, these differential immunization protocols may be an intriguing system for the analysis of the basic mechanism of the induction of helper and killer T lymphocytes to the transplantation antigens, and these may further contribute to a better understanding of the interrelation between cell-mediated and humoral immune responses.

In the present study, in order to establish the experimental conditions for the preferential generation of helper and killer T cells to the transplantation antigens, the T-cell responses toward the transplantation antigens of the two tumour cell lines were analysed in the allogeneic and syngeneic host combinations.

The results will show that when C57BL/6 mice were immunized with viable or mitomycin C-treated X-5563 plasmacytoma cells of C3H/He origin, both helper and killer T-cell activities were generated toward the alloantigens, whereas immunization with these allogeneic tumour cells killed by freezing and thawing procedure induced predominantly helper T-cell activity. In contrast, immunization of syngeneic C3H/He mice with above X-5563 plasmacytoma cells resulted in the preferential induction of killer T-cell activity to tumour-associated transplantation antigens (TATA), whereas the immunization of C3H/He mice with syngeneic MM102 mammary tumour cells generated almost exclusively helper T-cell activity to the corresponding TATA.

Thus, these results indicate that immunization with transplantation antigen(s) does not always result in generation of both the helper and killer T-cell activities, but a certain transplantation antigen system generates exclusively one type of T-cell activity, thus indicating that two distinct subsets of helper and killer T cells against transplantation antigen(s) can be induced independently without any collaboration between these different subsets.

MATERIALS AND METHODS

Mice

C3H/HeJ and C57BL/6J mice of both sexes, originally derived from the Jackson Laboratory (Bar Harbor, Maine) and maintained in our Institute, were used at 7–9 weeks of age.

Tumour cells

X-5563 plasmacytoma (PC) (Potter, Fahey and Pilgrim, 1957), and MM102 mammary tumour (MM) (Takizawa, Yamamoto and Mitsui, 1974), both derived from C3H/He strain and maintained by serial intraperitoneal passages into syngeneic C3H/He mice in the ascitic form, were utilized.

Induction of immunity against tumours

In allogeneic combination, C57BL/6 mice were immunized once or four times with intraperitoneal inoculations of 10^7 of PC cells in the following three different forms: one with viable cells, the other with tumour cells treated with mitomycin C (MMC) at a concentration of 40 μg/ml for 50 min at 37°, and the third with the tumour cells killed by freezing and thawing procedure.

In the syngeneic combination, C3H/He mice were intradermally inoculated with 10^6 of viable syngeneic PC or MM cells, followed by the surgical resection 7 days thereafter. By this procedure, tumour-specific immunity could be induced in more than 60 per cent of mice, and these immune mice manifested complete resistance against subsequent challenges with the corresponding tumour cell line.

Assay system for the killer T-cell activity

Cytotoxicity assay, originally devised by Hashimoto and Sudo (1971), was utilized with a slight modification into microculture-plate system. Radiolabelling of tumour cells was performed under rocking by incubation of a suspension of 3×10^6 of tumour cells in 3 ml of RPMI-1640 medium containing 10 per cent heat-inactivated foetal calf serum (RPMI-FCS) and 1 μCi of [^{3}H]uridine (the Radiochemical Center Ltd) (specific activity 2·9 Ci/mmol) in a Falcon 3002 tissue culture dish, at 37° for 60 min. After extensive washings, 3×10^4 of radiolabelled target tumour 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 ($75–300 \times 10^4$) of normal or immunized spleen cells suspended in 0·1 ml of RPMI-FCS. After effectors to targets interaction was proceeded for 20 h at 37° in 5 per cent CO_2/air, the cells in each well were harvested and washed twice with a large volume of cold 5 per cent 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 − c.p.m. in the immune well/c.p.m. in the normal well) × 100 per cent, where the normal or immune well denotes target cells incubated with normal or immune spleen cells. In the above cytotoxicity test, the final target cell viability in the normal wells was usually in the range of 80–100 per cent of that at the initiation of the assay.

Assay system for the helper T-cell activity

50×10^6 spleen cells from normal mice or the mice immunized with allogeneic or syngeneic tumours were transferred intravenously (i.v.) into 600 R X-irradiated syngeneic recipients along with 30×10^6 of DNP–KLH-primed spleen cells as DNP-specific B-cell source, and stimulated i.v. with 5×10^6 of trinitrophenylated tumour cells (TNP-PC or TNP-MM) as the challenging antigen.

The recipients were killed 7 days after cell transfer, and anti-DNP plaque-forming cells (PFC) in the recipients' spleens were enumerated by a modification of Cunningham's haemolytic plaque technique (Cunningham and Szenberg, 1968), as described previously DNP-substituted sheep erythrocytes as the indicator (Hamaoka, Yamashita, Takami and Kitagawa, 1975). The helper T-cell activity generated in the spleens of donor mice immunized with tumour cells was expressed by a helper index, denoting a ratio of the anti-DNP PFC to that of background response of B cells induced in the presence of non-immunized normal spleen cells.

Trinitrophenylation of tumour cells

Hapten-substituted tumour cells were prepared according to the method described by Philpott, Bower and Parker (1973). Briefly, 100×10^6 tumour cells pretreated with MMC at a concentration of 40 μg/ml for 50 min at 37° were suspended in 100 ml of Hanks's balanced salt solution containing 300 μg/ml of sodium 2,4,6-trinitrophenyl (TNP) sulphonate. After 30 min incubation at 37°, the tumour cells were washed twice and viability of the cells was determined by trypan blue dye exclusion test. By this system, more than 90 per cent of the tumour cells were viable after coupling with TNP.

Preparation of T cell-enriched population with nylon wool column

The procedure used was essentially the same as that described by Julius, Simpson and Herzenberg (1973). Briefly, nylon wool in LP-1 Leuko-Pak Leukocyte Filters (Fenwal Laboratories, Mortan Grove, Illinois) was soaked, dried and packed into a 10-ml syringe. After spleen cell suspensions were passed through a glass fiber column (obtained from Jintan Termo Company Ltd, Tokyo) to remove adherent and dead cells, they were loaded on the nylon wool column. The recovery of effluent cells was in the range of 15–20 per cent, and more than 90 per cent were Thy-1-positive, as determined by the susceptibility to anti-Thy-1 antiserum and guinea-pig complement treatment.

Statistical analysis

The numbers of PFC per spleen and the radioactivities in each well were logarithmically transformed, and means and standard errors calculated. Group comparisons were made employing Student's *t*-test. In those mice in which no PFC could be detected in the spleen, a value of 120/spleen was arbitrarily assigned to allow logarithmic transformation of the data, the value corresponding to the minimal number of PFC detectable in our assay.

RESULTS

T-cell responses of C57BL/6 mice immunized with various forms of allogeneic X-5563 tumour cells

C57BL/6 mice were immunized intraperitoneally with 10×10^6 of allogeneic X-5563 tumour cells (PC) one to four times at intervals of 7–10 days in the following forms, i.e. viable, MMC-attenuated, or killed by freezing and thawing procedure. Ten days after the last immunization, spleen cell suspensions were prepared from each group of mice, and killer and helper T-cell activities against the transplantation antigens on allogeneic tumour cells were measured. Killer T-cell activity was measured by *in vitro* cytotoxicity test and expressed as percentage specific killing. For a measurement of helper T-cell activity, 50×10^6 of spleen cells primed with various forms of X-5563 cells or unprimed spleen cells were transferred intravenously into 600 R X-irradiated recipients along with 30×10^6 of DNP–KLH-primed spleen cells as the DNP-primed B-cell source, and stimulated immediately thereafter with intravenous inoculation of 5×10^6 of MMC-treated TNP-PC cells. Seven days after the cell transfer and antigenic stimulation, indirect anti-DNP plaque-forming cells (PFC) were enumerated

Table 1. T-cell responses of C57BL/6 spleen cells immunized with various forms of allogeneic X-5563 plasmacytoma cells

Expt	Group	Spleen cell types tested*	Cytotoxic activity against X-5563 cells†		Helper T-cell activity against X-5563 cells‡	
			Target cell viability (per cent)	Specific killing (per cent)	Anti-DNP PFC per spleen	Helper index
1	A	Unprimed	80·4 (1·05)	0·0	1697 (1·41)	1·00
	B	Viable PC-primed (×1)	21·6 (1·05)§*	73·1	9158 (1·30)§***	5·40
	C	Killed PC-primed (×1)	84·4 (1·04)	0·0	3909 (1·05)§****	2·30
	D	MMC-PC-primed (×1)	85·8 (1·06)	0·0	7036 (1·27)§***	4·15
	E	MMC-PC-primed (×4)	23·7 (1·05)§*	70·5	20855 (1·04)§**	12·29
2	A	Unprimed	99·8 (1·07)	0·0	5250 (1·29)	1·00
	B	Viable PC-primed (×4)	31·6 (1·01)§*	67·3	59342 (1·14)§**	11·30
	C	Killed PC-primed (×4)	78·6 (1·09)§****	21·3	79859 (1·31)§**	15·21
	D	MMC-PC-primed (×4)	32·2 (1·04)§*	68·4	128654 (1·30)§*	24·51

* C57Bl/6 mice were i.p. immunized one to four times with 10^7 of either viable X-5563 cells (viable PC), freezed-thawed X-5563 cells (killed PC), or mitomycin C-treated X-5563 cells (MMC-PC), and spleen cells were submitted to cytotoxicity and helper T-cell assays 10 days after the last immunization.

† Cytotoxicity assay was performed at an effector to target cell ratio of 100:1. Percentage target cell viability was expressed as the geometric mean of triplicate cultures and the value in parentheses represents the standard error.

‡ Helper T-cell activity was measured by anti-DNP antibody response of transferred DNP–KLH-primed cells (30×10^6) to the stimulation of TNP-X-5563 cells (5×10^6) in the presense of spleen cells (50×10^6) from mice immunized with various forms of X-5563 cells. The helper index represents a ratio of mean value of PFC in experimental groups to that in unprimed group of each three to four animals.

§ Statistical significance against control: * $P < 0·001$, ** $P < 0·005$, *** $P < 0·05$, **** $0·10 < P < 0·20$.

in the spleens of those recipients, and helper T-cell activity present in those donor spleens was estimated by calculating a ratio of mean value of the PFC in the experimental group to that in unprimed group (helper index). Killer T-cell and helper T-cell activities generated in the spleens of C57BL/6 mice after the immunization with allogeneic X-5563 cells are summarized in Table 1.

As evident from comparison between Groups A and B in expts 1 and 2, one or four times immunizations with viable cells induced both of the killer and helper T-cell activities in parallel. A single immunization with killed allogeneic cells, as shown in group C in expt 1, did not induce any significant helper and killer T-cell activities, whereas repeated immunizations of killed PC cells as in group C in expt 2 induced relatively strong helper T-cell activity almost comparable to that in the repeated viable cell immunizations, with only a slight development of killer T-cell activity. On the other hand, the repeated immunizations with MMC-treated cells, being viable but non-replicating and the dose of antigen seemingly comparable to the killed cells, generated both killer and helper T-cell activities to almost the same extent as repeated immunizations of viable cells, as evident from comparisons between groups B and D in expt 2. However, the efficiency of MMC-PC immunization in inducing killer T-cell activity seemed to be relatively lower than the viable PC immunization, since a single immunization with MMC-PC induced only helper T-cell activity, without any detectable killer T-cell activity, as in group D in expt 1. This was, indeed, contrasted with the instance of repeated immunizations of MMC-PC in group E in expt 1, where striking killer and helper T-cell activities, such as in the viable cell immunization, were again observed.

Thus, these results indicate that C57BL/6 mice respond to viable allogeneic tumour cells of C3H/He origin with generation of both killer and helper T-cell activities, and seemingly preferential generation of helper T-cell activity was accomplished in some cases, such as repeated immunizations with killed allogeneic cells or a single immunization with MMC-treated cells. However, in this host–allogeneic tumour combination, no immunization regimen was found to develop preferential killer T-cell activity.

In the following section, in order to establish the immunization regimens for the preferential generation of helper or killer T cells to the transplantation antigens, the T-cell responses toward the tumour-

Table 2. Killer T-cell activity against X-5563 tumour cells in the spleens from syngeneic C3H/He mice at various immunization stages

Expt	Cytotoxicity* of spleen cells from:							
	i.d. Tumour-bearing mice†				Mice after surgical resection‡		Mice§ resistant against:	
	3 d	6 d	7 d	14 d	7 d	14 d	1st challenge	2nd challenge
1	—	—	52·9	47·6	34·1	24·9	—	51·1
2	0·0	23·3	32·4	—	—	—	37·1	29·6

* Cytotoxicity assay was performed at an effector to target cell ratio of 100:1. Cytotoxicity was expressed as percentage specific killing.

† C3H/He mice were inoculated i.d. with 10^6 viable X-5563 cells 3, 6, 7 or 14 days before the assay.

‡ X-5563 tumour-bearing mice had their tumours surgically removed 7 days after tumour implantation, and cytotoxicity assays were performed on the indicated days after surgical resection of the tumours.

§ C3H/He mice survived after the first and second i.p. challenges with 10^6 viable X-5563 cells 2 weeks apart, started on day 14 subsequent to surgical resection of the tumour. The cytotoxicity of the spleen cells from those resistant mice was measured 10 days after the first or second challenges with the tumour.

associated transplantation antigens were further analysed in the syngeneic host–tumour combinations.

T-cell responses of C3H/He mice to syngeneic X-5563 plasmacytoma

When viable X-5563 cells were inoculated into syngeneic C3H/He mice, followed by the surgical resection of the tumour, tumour-specific cell-mediated immunity could be demonstrated in the spleens of these mice by means of the *in vitro* cytotoxicity assay and the *in vivo* tumour neutralization test. In addition to the evidence that this immunity could not be developed in T cell-deprived mice induced by the treatment of rabbit anti-thymocyte serum subsequent to adult thymectomy, an increase in cytotoxic activity in T cell-enriched population (T-cell content was more than 90 per cent) passed through nylon wool column of the immune spleen cells indicated the involvement of killer T cells in this tumour immunity (H. Fujiwara, T. Hamaoka, Y. Nishino and M. Kitagawa, in preparation).

As evident from Table 2, in which the development of killer T-cell activity subsequent to tumour inoculation and surgical resection is summarized, a significant killer T-cell activity emerged in the spleen 7 days after intradermal inoculation with 10^6 tumour cells and gradually decreased following tumour resection, whereas challenge with 10^6 viable tumour cells subsequent to tumour resection resulted in increase in killer T-cell activity in those spleens.

While allogeneic C57BL/6 mice responded to the inoculation of viable X-5563 tumour cells with both developments of killer and helper T-cell activities against alloantigens, it was investigated whether C3H/He mice can also respond to syngeneic tumour with those two types of T-cell developments against tumour-associated transplantation antigens (TATA). To examine helper T-cell activity against TATA of X-5563 cells, DNP–KLH-primed spleen cells were transferred intravenously into 600 R X-irradiated syngeneic recipients as a source of DNP-primed B cells along with the above spleen cells immunized with syngeneic X-5563 cells or unprimed spleen cells, and stimulated with intravenous inoculation of 5×10^6 MMC-treated TNP–PC cells. Seven days after the cell transfer and antigenic stimulation, indirect anti-DNP PFC were determined in the spleens of those recipients. The results of two experiments are summarized in Table 3, no significant helper T-cell activity being detected in spleens of syngeneic C3H/He mice in both tumour-bearing and the hyperimmune state, despite positive killer T-cell activities, as confirmed from the same donor pool.

Despite failure to detect significant helper T-cell

Table 3. Failure to detect helper T-cell activity against syngeneic X-5563 cells in the spleen from C3H/He mice immunized with X-5563 cells

Expt	B cells	Spleen cell types tested for helper T-cell activity	Second antigen	Helper T-cell activity against X-5563 cells*	
				Anti-DNP PFC per spleen	Helper index
1	DNP–KLH-primed cells	Normal	5×10^6 TNP–PC	2755 (1·34)	1·00
		PC-tumour bearer†		1744 (1·38)	0·63
		PC-hyperimmune‡		872 (1·38)	0·32
2	DNP–KLH-primed cells	Normal	5×10^6 TNP–PC	< 120	1·00
		PC-tumour bearer†		< 120	1·00
		PC-hyperimmune‡		< 120	1·00

* Helper T-cell activity was measured by anti-DNP antibody response of transferred DNP–KLH-primed cells (30×10^6) at the stimulation of TNP-X-5563 cells (5×10^6) in the presense of the spleen cells (50×10^6) from mice immunized with syngeneic X-5563 cells or normal mice. The helper index represents a ratio of the mean value of PFC in experimental groups to that in normal groups of three to four animals.

† Mice were inoculated i.d. with 10^6 of X-5563 cells (PC) 10 days before. Mean cytotoxic activity as confirmed 3 days before the helper T-cell assay in the same donor pool was 39·7 per cent and 32·4 per cent in expts 1 and 2, respectively, at an effector to target cell ratio of 100:1.

‡ Mice resistant to consecutive i.p. challenges with 10^6, 3×10^6 and 5×10^6 viable X-5563 cells after surgical resection of i.d. implanted tumour. Mean cytotoxic activity, as confirmed 3 days before the helper T-cell assay in the same donor pool, was 46·9 per cent and 31·3 per cent in expts 1 and 2, respectively, at an effector to target cell ratio of 100:1.

activity in the above system, the fact remains that TATA on the X-5563 cells with which T lymphocytes will interact, was denatured or masked by TNP coupling. The positive interaction of TNP-PC cells with the alloantigen-sensitized helper T cells as shown in Table 1, however, may make this possibility a remote one, but it is not definitive whether this is also relevant to the TATA in the syngeneic system. The results in Table 4 which demonstrate that killer T cells against TATA were still capable of reacting with the TNP–PC to almost the same extent as with the uncoupled target cells (PC), clearly negate this possibility, and these, in turn, indicate that TATA was still intact after trinitrophenylation of X-5563 cells.

Table 4. Cell-mediated cytotoxicity* to TNP-substituted X-5563 cells by X-5563-immunized syngeneic killer T cells†

Target cells	Percentage specific killing	
	Expt 1	Expt 2
PC	46·1	65·9
TNP–PC	40·1	38·3

* Cytotoxicity assay was performed at an effector to target cell ratio of 100:1.

† Spleen cells from C3H/He mice which were resistant to i.p. challenge of 10^6 X-5563 cells (PC) after surgical resection of i.d. implanted tumour.

Thus, taken collectively, the results in Tables 2 and 3, the immunization of C3H/He mice with syngeneic X-5563 tumour cells generated preferentially killer T-cell activity to TATA.

T-cell responses of C3H/He mice to another syngeneic MM102 mammary tumour

In order to explore further the experimental conditions for the preferential generation of helper or killer T-cell activity against TATA, MM102 mammary tumour (MM) of C3H/He origin was utilized, and examined to see if in syngeneic C3H/He mice, the T-cell response against TATA could be shown to be those of helper cells or killer cells. The helper T-cell activity was determined in the spleen cells immunized with MM cells by the same experimental procedure as in X-5563 tumour system, except for the challenge with TNP-MM, and the results are summarized in Table 5. The results in expt 1 in Table 5 indicate that in MM102 tumour system strong helper T-cell activity was detected in the spleens of

Table 5. Helper T-cell activity against MM102-associated antigens in the spleens of C3H/He mice immunized with syngeneic MM102 tumour cells

Expt	B cells	Spleen cell types tested for helper T cell activity	Second antigen	Helper T-cell activity against MM or PC cells	
				Anti-DNP PFC per spleen	Helper index
1	DNP–KLH-primed cells	Normal		649 (1·51)	1·00
		MM–tumour bearer*	5×10^6 TNP–MM	7692 (1·05)	11·85
		MM–immune†		8544 (1·07)	13·16
		MM–hyperimmune‡		14,312 (1·19)	22·05
2	DNP–KLH-primed cells	Normal	5×10^6 TNP–MM	120 (1·00)	1·00
		MM–tumour bearer*	5×10^6 TNP–MM	3515 (1·33)	29·29
		Normal	5×10^6 TNP–PC	294 (1·45)	1·00
		PC–immune§	5×10^6 TNP–PC	120 (1·00)	0·41

* Mice were inoculated i.d. with 10^6 MM102 cells (MM) 13 and 14 days before, in expts 1 and 2, respectively.

† Mice which were resistant to i.p. challenge with 10^6 MM102 cells after surgical resection of i.d. implanted tumour.

‡ Mice which were resistant to consecutive i.p. challenges with 10^6, 3×10^6 and 5×10^6 MM102 cells after surgical resection of i.d. implanted MM102 tumour.

§ Mice which were resistant to i.p. challenge with 10^6 X-5563 cells (PC) after the surgical resection of i.d. implanted X-5563 tumour.

Table 6. Failure to detect significant cell-mediated cytotoxicity in the spleen of C3H/He mice immunized with syngeneic MM102 tumour cells

Expt	Effector cells from:	Effector cell types	Effector to target cell ratio	Percentage specific killing
1	C3H/He	Normal	100:1	—
		MM-tumour bearer*		0·0
		Normal	50:1	—
		MM-tumour bearer		0·0
		Normal	25:1	—
		MM-tumour bearer		0·0
2	C3H/He	Normal	100:1	—
		MM-immune†		10·8§***
		Normal	50:1	—
		MM-immune		0·0
		Normal	25:1	—
		MM-immune		2·9§***
3	C3H/He	Normal	50:1	–
		MM-immune†		0·0
		column-effluent immune T‡		18·4§*
	C57BL/6	Normal	50:1	—
		MM-immune†		8·3§***
		column-effluent immune T‡		48·8§**

* Mice inoculated i.d. with 10^6 of MM102 tumour cells 7 days before.

† Mice which resisted to i.p. challenge with 10^6 of MM102 tumour cells after the surgical resection of i.d. implanted tumour.

‡ Immune spleen cells were purified to T cell-enriched population through the nylon wool column. Thy-1 positivity of the effluent cells was more than 90 per cent.

§ Statistical significance against control: * $0{\cdot}05 < P < 0{\cdot}10$; ** $0{\cdot}01 < P < 0{\cdot}02$; *** $0{\cdot}20 < P$.

mice at various stages of immunization from tumour-bearing to the hyperimmune state, and helper T-cell activity seemed to increase corresponding to the extent of immunization. Furthermore, in expt 2, which was carried out in parallel with X-5563 plasmacytoma-immunized spleen cells, a significant helper T-cell activity was again detected in the spleens of MM102 tumour-bearing mice, whereas the helper T-cell activity against X-5563 TATA was not detected, this being consistent with the results in Table 3.

On the other hand, as shown in expts 1 and 2 in Table 6, in the spleens of MM102 tumour-bearing or MM102-immune mice where the strong helper T-cell activity was detected, no significant killer T-cell activity was observed against MM102 cells. This was further substantiated by the result in expt 3 which was conducted in parallel with allogeneic host–MM tumour combination. Killer T-cell activity in the syngeneic spleen could not rise to a statistically significant level even after purification of MM-immune cells to T cell-enriched population through the nylon wool column (T-cell content was more than 90 per cent), whereas the T cell-enriched population of the spleen cells from allogeneic MM–immune C57BL/6 mice exhibited apparently significant cytotoxicity against MM102 cells.

It is interpreted from the results in Tables 5 and 6 that the immunization of the syngeneic C3H/He mice with MM102 tumour cells preferentially generated helper T-cell activity against MM102-associated transplantation antigens.

DISCUSSION

In the present study, in order to establish the experimental conditions for the preferential generation of helper and killer T-cell activities towards transplantation antigens, the T-cell responses towards the transplantation antigens of the two tumour cell lines were investigated in the allogeneic and syngeneic tumour–host combinations. When C57BL/6 mice were repeatedly immunized with viable or MMC-treated allogeneic tumour cells, both induced the helper and killer T-cell activities against alloantigens, whereas repeated immunizations of C57BL/6 mice with these allogeneic tumour cells killed by a freezing and thawing procedure generated predominantly helper T-cell activity (Table 1).

In view of the preferential generation of killer and helper T-cell activities to alloantigens, the results presented here are partly discordant with the observations by Dennert and Lennox (1974) and Igarashi *et al.* (1975), in which they claimed that the immunization of allogeneic host with viable tumour cells induced exclusively the killer T-cell activity to the immunized allogeneic tumour cells without any detectable helper T-cell activity. They measured helper T cell activity by *in vitro* induction of anti-hapten antibody response, whereas we assessed it by adoptive transfer system of lymphoid cells into sublethally X-irradiated recipient mice. These discordant results may be explained by the difference in sensitivity of the two assay systems detecting the helper T-cell activity. Alternatively, it could be that helper T cells generated in the spleens of mice immunized with viable tumour cells were not detected because their activity was suppressed by a population of suppressor cells (Dennert *et al.*, 1974), or their detection was interfered with by destruction of antigenic tumour cells by cytotoxic killer T lymphocytes, especially in the *in vitro* system. However, as repeated injections with viable allogeneic cells into the animals consistently induced highly significant IgG alloantibody formation which could depend on helper T-cell generation (Klein, Livnat, Hauptfeld, Jerabek and Weissman, 1974), one may reasonably assume that helper T cells against alloantigens are generated by immunization with viable allogeneic tumour cells, but are unable to be detected in an *in vitro* assay because of the reasons outlined above. In contrast, our assay system of helper and killer T-cell activities, in which both activities could be detected in parallel, seems to be devoid of such complications.

On the other hand, consistent with their results, repeated immunizations with killed allogeneic tumour cells generated predominantly helper T-cell activity with barely detectable killer T-cell activity. For an explanation of this preferential generation of helper T cells by immunization with allogeneic tumour cells killed by a freezing and thawing procedure, the following possibilities may be considered. (1) Helper and killer T cells recognize the same spectrum of antigenic determinants, but differences may exist between the thresholds of the dose of antigen for both subsets of T cells to be effectively stimulated. The helper T cells are more effectively stimulated by a relatively low dose of antigen than the killer T cells, and immunization with the low dose of antigen, such as non-replicating dead cells, results in preferential induction of helper cells,

whereas immunization with a relatively high dose of antigen, such as replicating viable cells, induces both the helper and killer T-cell responses. However, the result obtained by repeated immunizations with MMC-treated allogeneic cells may negate this possibility, since MMC-treated cells, being non-replicating and therefore considered to be quantitatively the same as the killed cells, induced both the killer and helper T-cell activities comparable to those of the immunization with viable cells (expt 2 in Table 1). Thus, the difference in the T-cell types induced by the different immunization regimens may not be merely due to the difference in dose of immunizing antigen.

(2) Alternatively, the above selective T-cell response may result from the difference in the spectrum of antigenic determinants by which the killer and helper T lymphocytes are stimulated. Killer T-cell determinants are expressed on the viable immunizing cells, but not on killed tumour cells, probably due to destruction of the determinants by the killing procedure of the immunizing cells. On the other hand, helper determinants may be relatively resistant to that treatment and therefore expressed on both viable and killed cells.

In fact, as evident from the present analysis in syngeneic tumour–host combinations, the T-cell responses to the two syngeneic tumours revealed that TATA of X-5563 plasmacytoma induced preferentially killer T-cell activity with no detectable helper T-cell response (Tables 2 and 3), whereas TATA of MM102 mammary tumour generated predominantly helper T-cell activity with no or very weak killer T-cell response (Tables 5 and 6). Thus, these results clearly indicate that a certain transplantation antigen does not always induce both the helper and killer T-cell responses in parallel, but rather induces preferentially one of these responses. Our preliminary experiments, although not shown in the present study, also demonstrated that X-5563 plasmacytoma did not induce any significant cytotoxic antibody response, whereas MM102 tumour induced specific cytotoxic antibody against TATA. The preferential induction of the killer or helper T-cell activity against those two lines of tumours may also closely correlate with this phenomenon.

Wernet and Lilly (1975) reported that among B.10 congenic strains, the difference in the D region of H-2 locus induced only killer T-cell activity with no detectable helper T-cell activity, and similar observation was also made by Klein, Forman, Hauptfeld and Egorov (1975), in that the histocompatibility difference only in the K region of H-2 locus induced killer T-cell activity without any detectable alloantibody production which was conceivably dependent on the generation of helper T lymphocytes. Thus, taking together their results and ours, it is conceivable that there may be two distinct antigenic determinants specific for helper and killer T cells, and that viable allogeneic cells may carry helper and killer determinants on their cell surfaces to stimulate both cell types in parallel, whereas the two syngeneic tumour cells investigated here carry preferentially either helper or killer determinants.

In comparison of the immune resistance against syngeneic and allogeneic tumours, the latter system may provide double defense mechanisms of both cell-mediated and humoral immunity relating to the killer and helper T-cell generations. In contrast, as demonstrated in the present study, the immune response to the syngeneic tumour system does not always involve both the killer and helper T-cell activities. Thus, the immune response to the syngeneic tumour may be different from that to the allogeneic tumour, not only quantitatively but also qualitatively, and this qualitative difference may account, in part, for difficulty in rejection of the syngeneic tumour.

Finally, it may be relevant to discuss the preferential generation of the killer or helper T-cell activity as observed in the syngeneic system with respect to a possible cellular collaboration mechanism in the generations of these T-cell types. As a matter of fact, the above dissociative generation of killer or helper T cells against TATA indicated that the generation of helper T-cell activity against TATA may not be an absolute prerequisite for the effective development of killer T-cell response. However, these results do not, indeed, exclude a possibility of the augmenting effect of helper T cells on the generation of killer T-cell activity. Cantor and Boyse (1975b) reported that killer T-cell development against allogeneic tumour cells was augmented by the collaboration with other subset of amplifier T cells which carries the Ly specificity different from the killer T cells, and recognizes alloantigens different from those recognized by prekiller cells. At present, however, it is not certain whether their amplifier T cells are equivalent to the helper T cells in the present study, or to the proliferating cells in the mixed lymphocyte culture (MLC), but it is highly con-

ceivable that, if the collaborative response of helper or MLC-proliferating cells with the prekiller cells can be successfully induced in syngeneic tumour system, the T-cell response against killer determinants may be potentiated.

In this context, if animals are immunized with tumour cells modified by additional antigenic determinants with which the amplifier T cells may be capable to react, the augmentation of the specific T-cell response against killer determinants will be definitely expected. Our preliminary results which demonstrated that the mice preimmunized with hapten–autologous protein conjugate and consequently generated hapten-reactive T lymphocytes (Hamaoka *et al.*, 1975) exhibited the augmented killer T-cell development against both the alloantigens and TATA after immunization with hapten-substituted tumour cells, may be consistent with this notion. Further study concerning the T-cell type responsible for this amplification is under investigation.

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