IN VITRO INDUCTION OF TUMOR-SPECIFIC IMMUNITY

I. Parameters of Activation and Cytotoxic Reactivity of Mouse Lymphoid Cells Immunized In Vitro against Syngeneic and Allogeneic Plasma Cell Tumors*

By HERMANN WAGNER‡ and MARTIN RÖLLINGHOFF‡

(From The Walter and Eliza Hall Institute of Medical Research, Parkville, 3050, Victoria, Australia)

(Received for publication 3 January 1973)

The concept of a possible role of normal immune responses as a surveillance mechanism against malignant cell growth (1, 2) is based on evidence that most tumor cells are antigenic in the host of their origin (3–6). Unlike transplantable histoincompatible tumors, syngeneic tumors impose rather strict experimental difficulties as they evoke a type of immune response that is often marginal in activity and thus is usually measurable only in vivo. However, evidence is accumulating that immunity against syngeneic tumors is a cell-mediated immune response (7–9) and that the cell-mediated immune response is directed exclusively against tumor-associated transplantation antigens (TATA)[1] not present on the (original) normal cells.

Cell-mediated immune responses induced in dissociated lymphoid cell cultures against TATA would facilitate a deeper understanding of the cellular mechanisms underlying tumor immunity in syngeneic hosts. We have therefore attempted to immunize dispersed mouse lymphocytes in vitro against syngeneic antigenic plasma cell tumors (PCT). The purpose of this study was (*a*) to define the parameters of in vitro immunization of mouse lymphoid cells against plasma cell tumor TATA, (*b*) to test for specificity of the cytotoxic response obtained, and (*c*) to compare quantitatively syngeneic tumor immunity to PCT with allograft immunity. The results suggest that it is possible to activate mouse lymphocytes against TATA in vitro. A preliminary report has been given elsewhere (10).

---

* This work was supported by the National Health and Medical Research Council (Australia), U.S. National Institutes of Health grant AI-0-3958, and the Deutsche Forschungsgemeinschaft, and is pursuant to U.S. Public Health Service contract NIH-HCI-g-72-3889. It is publication no. 1852 from The Walter and Eliza Hall Institute.

‡ Supported by the Deutsche Forschungsgemeinschaft. Present address and reprint requests to: Institut of Medical Microbiology, University of Mainz, D-65 Mainz, Germany.

[1] *Abbreviations used in this paper:* CL, cytotoxic lymphocytes; FCS, fetal calf serum; FEM, fortified Eagle's medium; PCT, plasma cell tumors; SI, stimulation index; SRC, sheep red cell; TATA, tumor-associated transplantation antigens.

Materials and Methods

Mice.—Highly inbred CBA/H/Wehi (*H-2*^{k}), BALB/c (*H-2*^{d}) NZB (*H-2*^{d}), and C57BL/6 (*H-2*^{b}) mice were used throughout.

Preparation of Cortisone-Resistant Thymocytes.—Female CBA, BALB/c, or NZB mice 35–60 days old were injected intraperitoneally with 1.5 mg of cortisone acetate suspended in phosphate-buffered saline. 24 h later, the thymocytes were removed under sterile conditions. The corticosteroid injection resulted in a 75–85% depletion of thymus lymphocytes.

Cell Suspensions.—Mice were killed by cervical dislocation. The spleen and thymus were excised under aseptic conditions, minced, and strained through an 80-gauge stainless sieve into cold culture medium. To remove cell clumps, the cell suspensions were placed in a conical tube over 1 ml of fetal calf serum (FCS, Commonwealth Serum Laboratories, Parkville, Australia). The supernatant fraction, a single cell suspension, was then taken; and its viability was determined by the dye exclusion method (11).

Culture Medium.—Eagle's minimal medium with nonessential amino acids (Grand Island Biological Co., Grand Island, N. Y., catalog. no. F-15) was used. This was supplemented with 100 U/ml of penicillin G, 100 μg/ml of streptomycin, and 5% FCS and was buffered with sodium bicarbonate.

Culture System.—The culture system used is a modification (12) of that first described by Marbrook (13) and Diener and Armstrong (14) and has been used for the induction of in vitro allograft responses (12, 15). It consists basically of a glass tube (diameter 2.1 cm) sealed off at the bottom by a dialysis membrane and suspended from the stopper of an Erlenmeyer flask filled with about 50 ml of culture medium. 60×10^6 responder lymphocytes were cultured together with 2×10^6 allogeneic or syngeneic irradiated plasma tumor cells in a volume of 3 ml of culture medium in the inside of the glass tube. The cultures were incubated in 10% CO_2 in air at 37°C.

Cell-Mediated Cytotoxicity Assay.—The ^{51}Cr-release assay used was a modification of that described by Brunner et al. (16) and was performed as described previously in detail (12, 15).

Labeling of target cells: Usually $3–4 \times 10^6$ tumor cells were labeled with 100 μCi of chromate ^{51}Cr (CEA, Gif-sur-Yvette, France) in a final volume of 1 ml of fortified Eagle's medium (FEM, Grand Island Biological Co.) for 20 min at 37°C. The cells were washed twice through FCS and adjusted to a concentration of 10^5 cells/100 μl.

Assay: At termination of the culture, the cells from triplicate cultures were harvested, pooled, and washed twice. The viability of the cultured cells was determined by eosin exclusion. In the experiments in which the cytotoxic activity generated was quantitatively titrated, the cells were adjusted to 5×10^6 viable transformed lymphocytes/ml. A dilution of 1:6 and 1:36 was performed, and the cells were assayed for cytotoxic activity against a constant number of ^{51}Cr-labeled target cells. Thus 1 ml each of the respective cell dilutions was pipetted to 35×10 mm Petri dishes (Falcon Plastics, Inc., Los Angeles, Calif.), and 50 μl of the target cells (5×10^4 cells) were added, resulting in ratios of 100, 16.6, and 2.7 cytotoxic lymphocytes (CL) to one target cell. Each assay was performed in triplicate. The dishes were placed in an airtight box, gassed with 10% CO_2, and rocked on a platform for either 4 or 6 h. The cells were then harvested and transferred into plastic tubes; a drop of 5% sheep red cell (SRC) suspension was added, the solution was centrifuged, and the supernatant was separated from the pellet. The radioactivities of the supernatant and the pellet were determined in a Packard autogamma scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.), and the results were expressed as percent of maximal ^{51}Cr release as determined by freezing and thawing 5×10^4 labeled target cells four times.

"One Way" Mixed Culture.—The mitotic response of mouse lymphocytes against allogeneic or syngeneic irradiated plasma cell tumors was determined at a ratio of responder cells to stimulator cells of 30:1. The culture system used was that of Diener and Armstrong (14), as described in detail elsewhere (12). Responder cells (15×10^6) were cultured with 0.5×10^6 ir-

radiated tumor cells, thus keeping the cell density per surface area constant to the system in which cytotoxic activity was generated. At termination of the culture, the cells were pulsed with [^{3}H]thymidine (Radiochemical Centre, Amersham, England) for 7 h at a final concentration of 0.7 μCi/ml, and the acid precipitable radioactivity was determined according to the method of Wilson (17), as described in detail previously (12). The stimulation index (SI) compares the ratio of [^{3}H]thymidine incorporation of the combination responder cells plus stimulator cells with that of the controls (incorporation of responder cells alone and stimulator cells alone).

Lymphoid Tumor Cells.—Tumor cell lines HPC 108, HPC 6, and Wehi 22 were generously provided by Drs. N. L. Warner and A. W. Harris (The Walter and Eliza Hall Institute). Tumor cell lines EL4 (18) and P815 (19) were originally obtained from Drs. Brunner and Cerottini, Lausanne, Switzerland. All tumor lines were kept in continuous stationary suspension cultures by Dr. A. W. Harris.

HPC 108 is a mineral-oil-induced plasma cell tumor, induced in a BALB/c <—> C57BL allophenic mouse and shown by transplantation to be of BALB/c origin (*H-2*^{d}).[2] The results were confirmed by the demonstration that CBA cortisone-resistant thymocytes (*H-2*^{k}), activated in vitro (15) against C57BL (*H-2*^{b}) transplantation antigens, were efficient in lysing EL4 (*H-2*^{b}) target cells, but did not lyse HPC 108 target cells. However, when CBA cortisone-resistant thymocytes were activated against BALB/c (*H-2*^{d}) transplantation antigens, HPC 108 target cells were effectively lysed (Table I). HPC 6 is a mineral-oil-induced plasma cell tumor originated in a NZB (*H-2*^{d}) mouse.[3] Wehi 22 is a radiation-induced thymic lymphoma induced in a BALB/c (*H-2*^{d}) mouse.[3]

X-Irradiation.—Plasma cell tumors were irradiated with 4,000 rad in a Philips (RT 250) X-ray machine (Philips Electronic Instruments, Mount Vernon, N. Y.). Details of the irradiation technique for cells have been given by Miller and Sprent (20).

Mitomycin C Treatment.—Spleen cells were treated with mitomycin C (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 35 μg/ml for 30 min at 37°C and washed twice before being used as cell-bound alloantigen.

RESULTS

Cytotoxic Allograft Responses Against Myeloma Tumor Cells.—A first series of experiments was designed to titrate that cell concentration of irradiated (4,000 rad) allogeneic myeloma tumor cells per 60 $\times$ 10^6 responder cells that elicited optimal cytotoxic activity under the culture conditions used. Previous experiments had shown that with the use of allogeneic spleen cells as the source of cell-bound alloantigen, peak cytotoxic activity was obtained after a 6-day culture (12, 15). Thus 60 $\times$ 10^6 CBA cortisone-resistant thymocytes (*H-2*^{k}) were cultured together with a graded number of irradiated HPC 108 (*H-2*^{d}) tumor cells (or irradiated HPC 6 [*H-2*^{d}] tumor cells). After 6 days, cells from three cultures per group were pooled and assayed for cytotoxicity against the immunizing tumor cells in a ^{51}Cr-release assay. As illustrated in Fig. 1, the optimal cytotoxic allograft response was obtained at a ratio of responder cells to irradiated stimulator cells of about 30:1.

The specificity of cell-mediated cytotoxicity for sensitizing *H-2*^{d} alloantigens was established by demonstrating that CBA cortisone-resistant thymocytes (*H-2*^{k}) activated in vitro against *H-2*^{d} PCT were cytotoxic against *H-2*^{d} tumor

[2] Harris, A. W., M. C. Holmes, and N. L. Warner. Manuscript in preparation.

[3] Warner, N. L. Manuscript in preparation.

cells of various origin, but were not effective in destroying $H\text{-}2^b$ tumor target cells (Table II). However, cytotoxic lymphocytes activated against $H\text{-}2^b$ tumor cells effectively lysed $H\text{-}2^b$ target cells yet were not cytotoxic against $H\text{-}2^d$ target cells.

TABLE I

In Vitro Characterization of the H-2 Phenotype of HPC 108 Plasma Tumor Cells

CBA cortisone-resistant thymocytes ($H\text{-}2^k$) activated in vitro against	Specific lysis of target cells*		
	EL4 ($H\text{-}2^b$)	P815 ($H\text{-}2^d$)	HPC 108 ($H\text{-}2^d$)
	%	%	%
$H\text{-}2^b$ (C57BL spleen cells)	71 ± 1.9	19 ± 2.3	6 ± 3.2
$H\text{-}2^d$ (BALB/c spleen cells)	8 ± 1.9	84 ± 1.4	80 ± 2.9

* The ^{51}Cr test was performed at a ratio of CL to target cells of 50:1. CBA cortisone-resistant thymocytes ($H\text{-}2^k$) were cultured in vitro against mitomycin C-treated BALB/c spleen cells ($H\text{-}2^d$) or mitomycin C-treated C57BL spleen cells ($H\text{-}2^b$). After 6 days' culture, the in vitro generated cytotoxic activity was assayed using three different tumor cells as target cells. Percent specific lysis was calculated by subtracting the mean of background lysis from total lysis. Background lysis (in the presence of normal CBA thymocytes) was: EL4 = 12 ± 1.3%; P815 = 15 ± 0.9%; HPC 108 = 18 ± 2.5%. Assay time was 4 h.

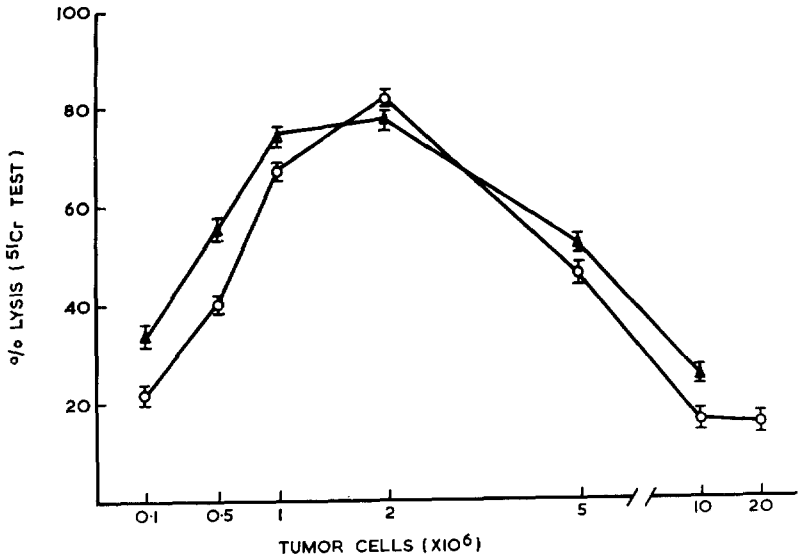

FIG. 1. Dose-response relationship of cytotoxic activity generated against allogeneic plasma cell tumor cells. 60 × 10^6 CBA cortisone-resistant thymocytes ($H\text{-}2^k$) were cultured with a graded number of irradiated HPC 108 ($H\text{-}2^d$) ○——○——○ or HPC 6 ($H\text{-}2^d$) ▲——▲——▲ plasma tumor cells. Cytotoxic activity generated in three cultures per group was assayed in a ^{51}Cr-release test, using the immunizing allogeneic plasma cell tumor cells as target cells. Background lysis (in the presence of normal thymocytes) was: HPC 108 = 20 ± 1.8%, HPC 6 = 24 ± 2.9%. Assay time was 4 h.

A kinetic study of cell-mediated cytotoxicity generated in vitro against cell-bound *H-2*^{d} alloantigens (irradiated HPC 108 cells or HPC 6 cells) was carried out (Fig. 2). Cytotoxic activity was first detected 3 days after culture. Peak activity was constantly observed at day 5–6, and dropped quickly thereafter. The kinetics of generation of cytotoxic anti-H-2 activity were found to be identical when CBA cortisone-resistant thymocytes and CBA spleen cells as responder cells were compared.

Cytotoxic Activity Against Syngeneic PCT.—Previous studies had demon-

TABLE II

Specificty of Cytotoxic Tumor Allograft Responses

CL activated in vitro against	Specific lysis of ^{51}Cr-labeled target cells*		
	EL4 (*H-2*^{b})	HPC 108 (*H-2*^{d})	HPC 6 (*H-2*^{d})
	%	%	%
HPC 108 (*H-2*^{d})	6 ± 2.3	82 ± 1.8	73 ± 1.9
HPC 6 (*H-2*^{d})	19 ± 3.4	70 ± 2.6	76 ± 1.7
EL4 (*H-2*^{b})	68 ± 2.1	7 ± 2.3	9 ± 3.6

* Ratio of CL to target cells of 50:1. Specific lysis was calculated by subtracting the mean of background lysis (in the presence of normal thymocytes) from total lysis obtained. Background lysis was: EL4 = 10 ± 2.3%; HPC 108 = 16 ± 1.2%; HPC 6 = 14 ± 3.1%. Assay time was 4 h.

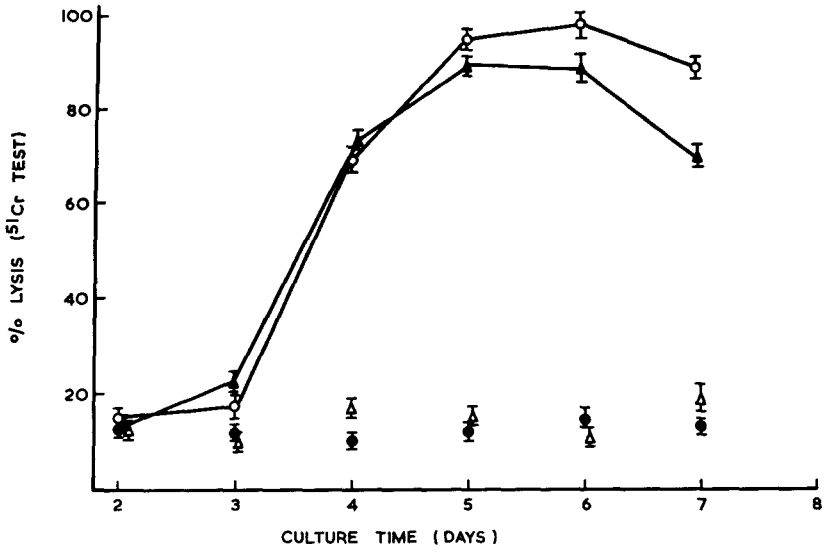

FIG. 2. Kinetics of the generation of cytotoxic tumor allograft responses. 60 × 10^6 CBA cortisone-resistant thymocytes were cultured together with either 2 × 10^6 irradiated HPC 108 cells ○——○——○ or 1.5 × 10^6 irradiated HPC 6 cells ▲——▲——▲. Cytotoxic activity was assayed in a ^{51}Cr-release test (4-h incubation time) at a ratio of CL to target cells of 50:1. The single points represent background lysis (in the presence of normal thymocytes).

strated that both HPC 108 PCT and HPC 6 PCT elicited in syngeneic animals tumor immunity with subsequent capacity of the host to reject the respective tumor (21). It was of interest, therefore, to investigate whether tumor immunity against these cell lines could be induced in vitro. From previous experience with in vitro allograft responses it was known that there is a critical dose-response relationship between the cell-bound alloantigen present in the culture and the cytotoxic response generated in a constant number of responder cells (22). Thus it was important to titrate out as first step in the syngeneic system the number of irradiated (4,000 rad) plasma tumor cells that presented an optimal antigenic stimulation to 60×10^6 syngeneic spleen cells per culture. As indicated in Table III, both tumor lines elicited in the respective syngeneic spleen lymphocytes a cytotoxic immune response in vitro. For 60×10^6 BALB/c spleen cells, a cell concentration of 2×10^6 HPC 108 cells yielded the highest cytotoxic immune responses (59% lysis of HPC 108 target cells). In the case of NZB spleen cells, almost the same concentration of the HPC 6 tumor cells was optimal (38% lysis of HPC 6 target cells).

Proliferative Response Against Syngeneic PCT.—Since a proliferative response precedes the generation of specific cytotoxic effector cells in cell-mediated allograft reactions (12, 23), a comparison of the mitotic response elicited by syngeneic PCT to that elicited by allogeneic PCT was performed. The "mixed culture" was set up at the ratio of responder cells to stimulator cells (30:1) that produced the highest cytotoxic activity. The results given in Table IV indicate that in the "allogeneic mixture" the highest SI was obtained at day 3 (SI > 5), whereas in the "syngeneic mixture" the SI obtained was much lower (SI < 2).

TABLE III

Dose-Response Relationship of Cytotoxic Activity Generated Against Syngeneic Plasma Tumor Cells

Syngeneic PCT per culture	Lysis of syngeneic ^{51}Cr-labeled PCT	
	HPC 108* target cells	HPC 6‡ target cells
$\times 10^6$	%	%
0.1	19 ± 2.5	21 ± 1.5
0.5	27 ± 3.8	25 ± 3.6
1	42 ± 1.9	38 ± 0.6
2	59 ± 2.6	36 ± 1.4
5	31 ± 2.5	25 ± 1.6
10	18 ± 0.9	22 ± 1.4

The cells harvested per group were assayed for cytotoxicity against the syngeneic tumor cells in a ^{51}Cr-release assay. Assay time was 6 h. Background lysis (in presence of normal spleen cells) of HPC 108 cells was 18 ± 1.7%; of HPC 6 cells, 16 ± 0.6%.

* BALB/c spleen cells (60×10^6) were cultured together with graded numbers of irradiated syngeneic HPC 108 PCT for 6 days.

‡ NZB spleen cells (60×10^6) were cultured together with graded numbers of irradiated syngeneic HPC 6 PCT for 6 days.

TABLE IV

Proliferative Response Against Syngeneic and Allogeneic PCT

Stimulator cells	Pulsing time	[^{3}H]Thymidine uptake					
		Syngeneic combination	Control	SI	Allogeneic combination	Control	SI
		cpm	*cpm*		*cpm*	*cpm*	
HPC 108	Day 3	23,000 ± 5,400	17,400	1.9	95,500 ± 16,700	17,590	6.0
(*H-2^d*)	Day 5	39,160 ± 4,900	13,600	3.1	33,060 ± 2,300	11,400	2.9
HPC 6	Day 3	17,700 ± 2,800	12,700	1.4	77,200 ± 11,800	14,300	5.4
(*H-2^d*	Day 5	35,600 ± 7,400	13,200	2.7	28,800 ± 4,300	13,100	2.2

Irradiated HPC 108 stimulator cells were cultured either together with allogeneic responder cells (CBA spleen cells, *H-2^k*) or with syngeneic responder cells (BALB/c spleen cells, *H-2^d*). Irradiated HPC 6 stimulator cells were cultured either with allogeneic responder cells (CBA spleen cells, *H-2^k*) or with syngeneic spleen cells (NZB spleen cells, *H-2^d*). [^{3}H]Thymidine incorporation was determined at day 3 and day 5 of culture after a 7-h labeling period.

However, it was noted that at day 5 the SI obtained in the "syngeneic mixture" was as high as in the "allogeneic mixture" (SI 2–3).

Kinetics of Induction of Tumor Immunity In Vitro.—A comparison of the kinetics of the generation of cytotoxic effector cells against syngeneic PCT with that against allogeneic PCT was carried out. In these experiments the PCT HPC 108 was chosen as a source of stimulator cells. The results obtained are illustrated in Fig. 3. Clearly, cytotoxic allograft responses were generated earlier in time than cytotoxic responses against TATA. For example, at day 3–4 significant allograft responses were obtained; they reached a peak at day 6. In the syngeneic "mixed culture" there was a lack of cytotoxic activity till day 4. However on days 5 and 6 an exponential increase in cytotoxic activity occurred, reaching a peak at day 7. Thus there appeared to be a separation in time between the generation of allograft immunity and tumor immunity in vitro.

Responsiveness of Cortisone-Resistant Thymocytes Against TATA.—Tumor immunity is thought to be a cell-mediated immune response and as such to be thymus dependent. As murine spleen cells are a mixture of both bone marrow-derived (B) lymphocytes and thymus-derived (T) lymphocytes, it was important to establish whether T lymphocytes alone could mount a cytotoxic immune response against TATA in vitro. Since cortisone-resistant thymocytes were efficient in cytotoxic in vitro allograft responses (15), the capacity of cortisone-resistant BALB/c thymocytes to mount a tumor response in vitro against syngeneic HPC 108 PCT was investigated. As shown in Fig. 4, cortisone-resistant thymocytes alone generated antitumor cytotoxic activity. Specific ^{51}Cr release increased with increasing ratios of CL to target cells over a range of 2:1 to 100:1.

Specificity of the Cytotoxic Antitumor Response.—The specificity of the cytotoxic response against syngeneic tumor cells was investigated. BALB/c corti-

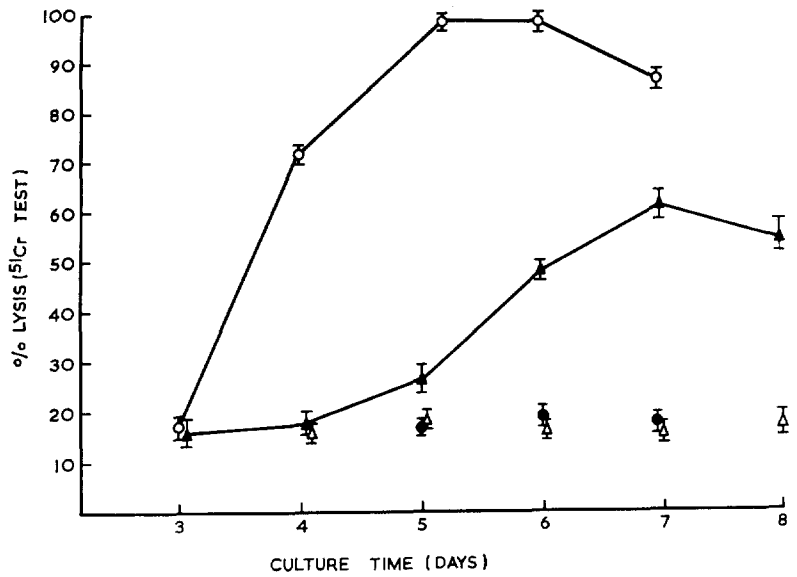

FIG. 3. Comparison of the kinetics of in vitro generation of cytotoxicity against allogeneic and syngeneic PCT. 60 × 10^6 CBA spleen cells (*H-2*^{k}) were cultured together with 2 × 10^6 irradiated HPC 108 PCT (*H-2*^{d}) ○——○——○——○. 60 × 10^6 BALB/c spleen cells (*H-2*^{d}) were cultured together with 2 × 10^6 irradiated syngeneic HPC 108 (*H-2*^{d}) PCT ▲——▲——▲. The cytotoxic activity generated was quantitated each day at a ratio of CL to target cells of 50:1 in a ^{51}Cr-release assay using the immunizing PCT as target cells. Assay time was 6 h. The single points represent background lysis.

sone-resistant thymocytes were activated against HPC 108 tumor cells, and the effector cells were assayed for cytotoxic activity against a variety of tumor cells of identical *H-2* phenotype and against allogeneic EL4 (*H-2*^{b}) target cells (Table V). BALB/c-derived cytotoxic lymphocytes activated against HPC 108 tumor cells were found to be specifically cytotoxic against HPC 108 target cells. However, in all experiments a small but significant background activity was noted against the other tumor cells tested. This unspecific cytotoxic activity was also apparent when responder cells were cultured in the presence of autologous irradiated spleen cells. Similar results were obtained when NZB cortisone-resistant thymocytes activated against the syngeneic PCT HPC 6 were tested for specificity (Table V). In the case of HPC 6 cells, the background activity was even more pronounced.

Comparison of Cytotoxic Responses Obtained Against Allogeneic PCT to That Against Syngeneic PCT.—The cytotoxic immune response obtained in vitro against histoincompatible PCT was compared with that obtained against syngeneic PCT. Once, CBA cortisone-resistant thymocytes (*H-2*^{k}) were activated against the allogeneic PCT HPC 108. To the other, the same number of BALB/c cortisone-resistant thymocytes (*H-2*^{d}) were activated against the syngeneic PCT HPC 108. After 7 days' culture, the cytotoxic activity generated

was assayed in vitro under identical conditions using ^{51}Cr-labeled HPC 108 cells as target cells. The results given in Fig. 5 illustrate that the magnitude of the cytotoxic immune responses generated against HPC 108 target cells was about 30-fold higher when the antigenic difference between responder cells and stimu-

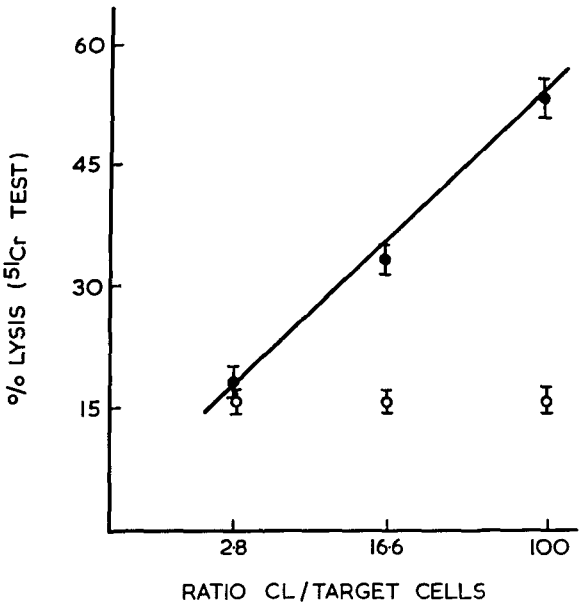

FIG. 4. Capacity of cortisone-resistant thymocytes to mount cytotoxic immune responses against syngeneic PCT. 60 × 10^6 BALB/c cortisone-resistant thymocytes were cultured together with 2 × 10^6 irradiated syngeneic HPC 108 tumor cells for 7 days. Cells were harvested, washed, and assayed for cytotoxic activity at various ratios of CL to target cells against ^{51}Cr-labeled HPC 108 cells. Assay time was 6 h.

TABLE V

Specificity of the Cytotoxic Response Against Syngeneic PCT

Target cells	Specific lysis of ^{51}Cr-labeled target cells*			
	BALB/c cortisone-resistant thymocytes activated against syngeneic		NZB cortisone-resistant thymocytes activated against syngeneic	
	HPC 108 PCT	Spleen cells	HPC 6 PCT	Spleen cells
	%	%	%	%
HPC 108 (*H-2^d*)	51 ± 1.4	12 ± 1.8	18 ± 1.2	14 ± 1.3
HPC 6 (*H-2^d*)	7 ± 2.3	9 ± 2.6	39 ± 2.4	11 ± 2.7
P815 (*H-2^d*)	11 ± 1.9	13 ± 0.8	20 ± 0.4	15 ± 2.1
EL4 (*H-2^b*)	6 ± 1.7	5 ± 1.3	10 ± 2.2	9 ± 1.1

* Ratio of CL to target cells of 50:1. Specific lysis was calculated by subtracting the mean of background lysis (in the presence of normal thymocytes) from total lysis obtained. Background lysis was: HPC 108 = 16 ± 1.3%; HPC 6 = 13 ± 0.6%; P815 = 17 + 0.6%; EL4 11 ± 2.0%. Assay time was 6 h.

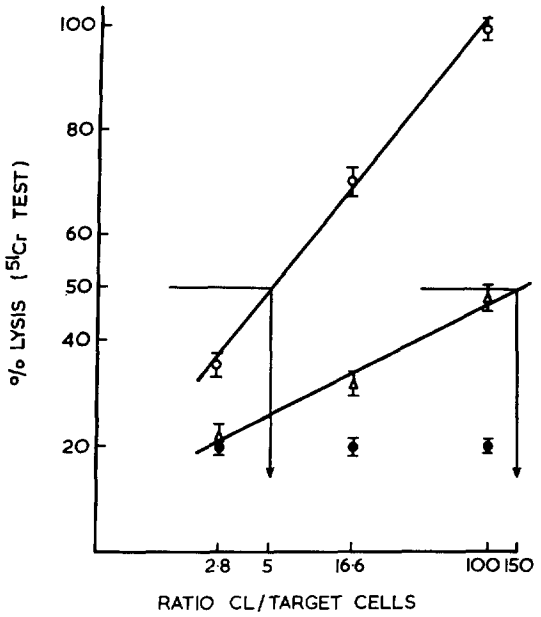

FIG. 5. Comparison of the cytotoxic activity generated against allogeneic and syngeneic PCT. 60 × 10^6 CBA cortisone-resistant thymocytes (*H-2*^{k}) were cultured together with 2 × 10^6 irradiated allogeneic HPC 108 cells (*H-2*^{d}) ○——○——○. The same number of BALB/c cortisone-resistant thymocytes (*H-2*^{d}) were cultured together with 2 × 10^6 irradiated syngeneic HPC 108 cells △—△—△. After 7 days' culture, the CBA-derived cells were harvested and washed, and a dilution was performed, resulting in a ratio of CL to target cells of 100:1, 16.6:1, and 2.8:1. The same number of cultures derived from BALB/c cells were harvested, pooled, and adjusted to the same volume as the CBA-derived cells and were similarly diluted in order to correlate the magnitude of the cytotoxic response against syngeneic PCT with that against allogeneic PCT. The assay time of the ^{51}Cr test was 6 h. The ratio of CL to target cells necessary to obtain 50% of lysis was compared. The single points represent background lysis of HPC 108 target cells.

lator cells included transplantation antigens of the major histocompatibility locus.

DISCUSSION

The data presented in this report support three broad conclusions. First, tumor immunity can be generated in vitro against syngeneic PCT and measured quantitatively in a ^{51}Cr-release assay. Second, there appear to be quantitative differences between in vitro cytotoxic responses directed against normal transplantation antigens and cytotoxic antitumor responses. Third, whereas the specificity in cytotoxic allograft reactions is directed against phenotypically expressed transplantation antigens, the specificity of cell-mediated antitumor responses appears to be restricted to antigens present only on the immunizing

syngeneic PCT. Such antigens seem not to be related to the major histocompatibility complex.

Several considerations led to the assumption that the use of murine plasma cell tumors may result in a suitable in vitro model for induction of syngeneic tumor immunity. It has previously been shown that both HPC 108 and HPC 6 PCT were antigenic in vivo, that is, they induced immunity in syngeneic hosts that could be transferred by spleen cells (21). In addition, both tumor lines were found to be suitable target cells in ^{51}Cr-release assays, i.e., they were easily lysed by cytotoxic lymphocytes activated in vivo against the H-2 phenotype of the plasma cell tumors (unpublished results). These results suggested that if cytotoxic tumor immunity was generated in vitro against syngeneic HPC 108 or HPC 6 tumor cells, it might be possible to detect the cytotoxic effect in a short-term ^{51}Cr-release assay. Finally, no evidence had been so far obtained that PCT induced a detectable humoral antibody response in syngeneic hosts (21). Thus it was reasonable to assume that antitumor activity generated in vitro might reflect mainly a cell-mediated immune response.

The parameters of in vitro tumor allograft responses against HPC 108 and HPC 6 were established. The kinetics of induction of cytotoxic responses and the specificity of the cytotoxic effector cells generated were similar to those obtained with the use of allogeneic spleen cells as stimulator cells in the induction phase of in vitro allograft responses (Tables I, II, references 12, 15). In both cases, the detectable cytotoxic response was directed against antigens of the major histocompatibility complex. For example, allogeneic PCT of the same *H-2* specificity as the immunizing tumor cells were also found to be effectively lysed (Table II). Thus the cytotoxic response generated reflected the characteristics of transplantation immunity (24) and as such was not due to antigens associated only with the PCT tumor cells.

The concept has been proposed that malignant cells have antigenic qualities distinct from those of the cell type from which they are derived (1–6). Since there is a lack of reactivity against self-transplantation antigens in normal organisms, one would predict that cocultivation of cortisone-resistant mouse thymocytes with syngeneic plasma tumor cells would result in an immune response directed solely against such antigenic differences between reactive cells and tumor cells as are recognized as foreign. Indeed, cytotoxic activity against syngeneic murine plasma cell tumors could be generated in vitro, and it appeared that a critical ratio of spleen cells to irradiated syngeneic tumor cells was necessary to provoke such a response (Table III). The cytotoxic activity generated was specifically directed against the antigenic syngeneic tumor cells in the sense that syngeneic tumor cells were lysed far more efficiently than other *H-2* phenotypically identical or nonidentical tumor cells. It was noted that although specificity for plasma cell TATA could be demonstrated, there was a low but significant lysis of other *H-2* identical tumor cells (Table V). Furthermore, it was found that cortisone-resistant thymocytes cultured in the presence of ir-

radiated syngeneic spleen cells alone appeared to acquire a certain degree of unspecific cytotoxic potential against tumor target cells. The magnitude of the unspecific cytotoxicity obtained when autologous spleen cells were used as antigen was found to be similar to the unspecific lysis noted in the case of cytotoxic effector cells activated against syngeneic tumor cells. The results obtained therefore suggested that in vitro activation of cortisone-resistant thymocytes against syngeneic plasma cell tumors resulted in two different effector stages, one specifically directed against plasma cell TATA, and one resulting in unspecific killing of the tumor target cells so far tested. Recent studies of the nature of the specific effector cells have provided evidence that the specificity of the cytotoxic reaction against TATA is a function of θ-positive lymphocytes, a characteristic of T cells.[4] Whether the unspecific killing is due to cross-reacting antigens on the target cells tested or to nonspecific killing of antigenically unrelated tumor cells, for example by macrophages (25), has to be analyzed.

Several techniques, for example, colony inhibition (26, 27), lymphocyte transformation (28), cytotoxicity (29), and cell migration with peritoneal exudate cells and peripheral leukocytes (30–32), have been applied in studying cell-mediated immunity against syngeneic tumors. Whereas lymphocyte transformation has been used as an in vitro correlate to the antigen recognition phase of lymphocytes reactive against TATA, inhibition of cell migration by immune lymphocytes has been used as a correlate to the state of tumor immunity, and colony inhibition or cytotoxicity has been employed to detect effector cells in tumor immunity. Since in the present investigation both the afferent and the efferent limbs of a cell-mediated immune response against plasma tumor cell TATA were induced in vitro and the effector cell activity was measurable in a direct cytotoxicity assay, the cellular mechanism involved could be analyzed.

The results obtained so far have been consistent with the view that tumor allograft immunity and tumor immunity against TATA of PCT are of the same general quality, that is, a cell-mediated function of T lymphocytes. Similarly to in vitro allograft responses (12, 15), a proliferative response preceded the appearance of cytotoxic effector cells against syngeneic PCT, although the magnitude of cell proliferation was low and cytotoxicity appeared late compared with responses against transplantation antigens (Table III, Fig. 3). Corticosteroid-resistant thymocytes alone were efficient in mediating both cytotoxic allograft responses and specific cytotoxic antitumor responses (Fig. 4). Finally, on a semilogarithmic scale, a nearly straight-line dose-response curve depicted the action of an increasing number of cytotoxic T lymphocytes on a fixed number of target cells over a wide range in both a tumor allograft system and a syngeneic tumor system (Fig. 5). This finding suggests that the mechanism of

[4] Röllinghoff, M., and H. Wagner. In vitro induction of tumor-specific immunity. II. The requirements for T lymphocytes and the protective potential against tumor growth in vivo. Manuscript submitted to *Eur. J. Immunol.*

specific in vitro cytotoxicity against syngeneic plasma tumor cells is similar to that against allogeneic target cells (15).

The immunogenicity of PCT TATA was found to be much weaker than that of normal transplantation antigens. PCT TATA induced in syngeneic cortisone-resistant thymocytes gave about a 30-fold lower cytotoxic response compared with that of transplantation antigens. Cytotoxic anti-TATA activity was found to appear later in time than cytotoxicity against *H-2* alloantigens (Fig. 4). Whether this difference in the induction of cytotoxicity is due to differing "antigenic strength" or to a different number of cells responding to the recognizable "antigenic units" has to be investigated.

Our studies suggest that HPC 108 and HPC 6 PCT are antigenic in vitro and provoke a cytotoxic cell-mediated immune response in syngeneic lymphocytes. However, it has to be stressed that the experimental conditions in the PCT system used facilitated the detection of cytotoxic immune responses. Besides the demonstration that these tumors were immunogenic in vivo (21), the facts that they grew in vitro in single cell suspension, were easy to label with ^{51}Cr-chromate, and were susceptible to the lytic effect of cytotoxic lymphocytes favored the results obtained. Thus, before general conclusions concerning the efficacy of in vitro immunization against TATA are allowed, it is necessary to demonstrate that other types of malignant cells are also immunogenic in in vitro culture systems.

SUMMARY

Induction of tumor-specific immunity in vitro was accomplished by cocultivation of cortisone-resistant murine thymocytes or spleen cells with irradiated syngeneic plasma cell tumors (PCT). The cytotoxic activity generated could be detected in a short-term ^{51}Cr-release assay. Optimal cytotoxic activity against PCT-associated transplantation antigens (TATA) was generated after 7 days in culture. Unlike cytotoxic responses to tumor allografts in which the cytotoxic activity was directed against allogeneic transplantation antigens, the cytotoxic activity obtained in the syngeneic tumor system was specific to the immunizing syngeneic PCT.

Similar parameters of induction of cytotoxic responses in in vitro tumor allograft responses and in the syngeneic tumor system suggested that both reactions are cell-mediated cytotoxic immune responses. With regard to the magnitude of cytotoxic responses obtained, allogeneic transplantation antigens induced about a 30-fold higher cytotoxic immune response than plasma cell TATA. The results are consistent with the concept that in vitro tumor allograft responses and in vitro responses against TATA of PCT are similar in quality, but differ in the magnitude of the cytotoxic response provoked.

We thank Drs. N. Warner and A. Harris for supplying the tumor cells used and Professor G. J. V. Nossal for stimulating discussions during the course of this work. The technical assistance of Helen Clark and Glenda Cousin is gratefully acknowledged.

REFERENCES

1. Burnet, F. M. 1957. Cancer: a biological approach. *Br. Med. J.* **1:**779.
2. Thomas, L. 1959. *In* Cellular and Humoral Aspects of the Hypersensitive States. H. S. Lawrance, editor. Casell, London. 529.
3. Foley, E. J. 1953. Antigenic properties of methylcholanthrene-induced tumors in mice of the strain of origin. *Cancer Res.* **13:**835.
4. Klein, G., H. O. Sjögren, E. Klein, and K. E. Hellström. 1960. Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary host. *Cancer Res.* **20:**1561.
5. Prehn, R. T., and J. H. Main. 1957. Immunity to methylcholanthrene-induced sarcomas. *J. Natl. Cancer Inst.* **18:**769.
6. Law, L. W. 1970. Studies of tumor antigens and tumor specific immune mechanisms in experimental systems. *Transplant. Proc.* **2:**117.
7. Hellström, I., K. E. Hellström, and H. O. Sjögren. 1970. Serum-mediated inhibition of cellular immunity to methylcholantrene-induced murine sarcomas. *Cell. Immunol.* **1:**18.
8. Oettgen, H. F., L. J. Old, E. P. McLean, and E. A. Carswell. 1968. Delayed hypersensitivity and transplantation immunity elicited by soluble antigens of chemically inbred tumors in inbred guinea pigs. *Nature (Lond.).* **220:**295.
9. Churchill, W. H., Jr., H. J. Rapp, B. S. Kronmann, and T. Borsos. 1968. Detection of antigens of a new diethylintrosamine-induced transplantable hepatoma by delayed hypersensitivity. *J. Natl. Cancer Inst.* **41:**13.
10. Wagner, H., and M. Röllinghoff. 1973. Cell-mediated immunity *in vitro* against syngeneic mouse plasma cell tumors. *Nat. New Biol.* **241:**53.
11. Hanks, J. H., and J. H. Wallace. 1950. Determination of cell viability. *Proc. Soc. Exp. Biol. Med.* **98:**188.
12. Wagner, H., and M. Feldmann. 1972. Cell-mediated immune response *in vitro*. I. A new *in vitro* system for the generation of cell-mediated cytotoxic activity. *Cell. Immunol.* **3:**405.
13. Marbrook, J. 1967. Primary immune response in cultures of spleen cells. *Lancet.* **2:**1279.
14. Diener, E., and W. D. Armstrong. 1969. Immunological tolerance in vitro: kinetic studies at the cellular level. *J. Exp. Med.* **129:**591.
15. Wagner, H., A. W. Harris, and M. Feldmann. 1972. Cell-mediated immune response *in vitro*. II. The role of thymus and thymus-derived lymphocytes. *Cell. Immunol.* **4:**39.
16. Brunner, K. T., J. Mauel, H. Rudolf, and B. Chapius. 1970. Studies of allograft immunity in mice. I. Induction, development and *in vitro* assay of cellular immunity. *Immunology.* **18:**501.
17. Wilson, D. B. 1967. Quantitative studies on the mixed lymphocyte interaction in rats. I. Conditions and parameters of response. *J. Exp. Med.* **126:**625.
18. Gorer, P. A., and D. B. Amos. 1956. Passive immunity in mice against C57BL leukosis EL4 by means of iso-immune serum. *Cancer Res.* **16:**338.
19. Dunn, T. B., and M. Potter. 1956. Transplantable mast cell neoplasm in the mouse. *J. Natl. Cancer Inst.* **18:**587.
20. Miller, J. F. A. P., and J. Sprent. 1971. Cell-to-cell interaction in the immune re-

sponse. VI. Contribution of thymus-derived cells and antibody-forming cell precursors to immunological memory. *J. Exp. Med.* **134:**66.

21. Röllinghoff, M., B. T. Rouse, and N. L. Warner. 1973. Tumor immunity to murine plasma cell tumors. I. Tumor-associated transplantation antigens of NZB and BALB/c plasma cell tumors. *J. Natl. Cancer Inst.* **50:**159.
22. Wagner, H. 1973. Cell-mediated immune response *in vitro*. IV. Metabolic studies of cellular immunogenicity. *Eur. J. Immunol.* **3:**84.
23. Wagner, H. 1972. The correlation between the proliferative and the cytotoxic responses of mouse lymphocytes to allogeneic cells *in vitro*. *J. Immunol.* **109:**630.
24. Batchelor, J. R., and L. Brent. 1972. Histocompatibility in transplantation immunity. *In* Immunogenicity. Felix Borek, editor. North Holland Research Monographs, Frontiers of Biology. **25:**409.
25. Evans, R., and P. Alexander. 1970. Co-operation of immune lymphocytes with macrophages in tumor immunity. *Nature* (*Lond.*). **228:**620.
26. Hellström, I., and K. G. Hellström. 1967. Cell-bound immunity to autologous and syngeneic mouse tumors induced by methylcholanthrene and plastic discs. *Science* (*Wash. D.C.*). **156:**981.
27. Hellström, I., K. E. Hellström, G. E. Pierce, and A. H. Bill. 1968. Demonstration of cell-bound and humoral immunity against neuroblastoma cells. *Proc. Natl. Acad. Sci. U.S.A.* **60:**1231.
28. Stjernsward, J., L. E. Ålmgard, S. Fransen, T. von Schreeb, and L. B. Waldström. 1970. Tumor-distinctive cellular immunity to renal carcinoma. *Clin. Exp. Immunol.* **6:**963.
29. Kikuclir, K., Y. Kikuclir, M. G. Philipps, and C. M. Southam. 1972. Tumor-specific, cell-mediated immune resistance to autochthonous tumors. *Cancer Res.* **32:**516.
30. Bloom, B. R., B. Bennett, I. H. Oettgen, E. McLean, and L. J. Old. 1969. Demonstration of delayed hypersensitivity to soluble antigens of chemically induced tumors by inhibition of macrophage migration. *Proc. Natl. Acad. Sci. U.S.A.* **64:**1176.
31. Halliday, W. J. 1971. Blocking effect of serum from tumor-bearing animals on macrophage migration inhibition with tumor antigens. *J. Immunol.* **106:**855.
32. Vaage, J., D. R. Jones, and B. W. Brown. 1972. Tumor specific resistance in mice detected by inhibition of macrophage migration. *Cancer Res.* **32:**680.