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Cellular Cytotoxicity and Serum Inhibition in Normal Mice following Transfer of Xenogeneic Tumor-sensitized Cells[1]

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ABSTRACT

Findings from previously reported investigations revealed that lymphoid and myeloid cells from tumor-bearing mice, when transferred to normal syngeneic mice never exposed to a tumor, imparted "information" which resulted in the production of tumor-specific cytotoxic cells by recipients. The present studies determined the cytotoxicity of cells from normal mice which were recipients of cells obtained from rats (xenogeneic) sensitized to mouse tumor. Normal rat lymph node cells (LNC) or spleen cells (SPC), when evaluated prior to their transfer, were found to be noncytotoxic to tumor target cells. LNC or SPC from rats sensitized to mouse tissue, either normal or tumor, were highly cytotoxic. Subsequent to the transfer of LNC or SPC from normal rats or from those sensitized to H-2 antigen (normal mouse tissue), little or no cytotoxicity was identified in LNC, SPC, or macrophages cultured from bone marrow cells of normal recipient mice. When the transferred cells were derived from rats sensitized to both H-2 and tumor antigen, *i.e.*, tumor cells, and the target cells were from the same tumor used for sensitization, maximal cytotoxicity was demonstrated in cultured macrophages, LNC, and SPC of normal mouse recipients. An increase in cellularity of recipient nodes, spleen, and bone marrow occurred following transfer of tumor-sensitized xenogeneic cells, unsensitized rat cells, or those cells sensitized to normal mouse spleen, indicating an equivalent recruitment of host cells by all types of xenogeneic cells transferred. The behavior of the recruited cells, *i.e.*, tumor cytotoxicity, was entirely dependent upon the use of tumor for sensitization of donor cells. Findings similar to those in the syngeneic system indicate that sera from normal cell recipients inhibit the cytotoxicity of cells derived from tumor-bearing animals. The findings indicate that information has been transferred by tumor-sensitized xenogeneic cells to normal animals that have never been exposed to tumor cells, which results in their production of tumor-specific cytotoxic cells. H-2-sensitized xenogeneic cells failed to produce such an effect. The relation of these findings to the use of xenogeneic cells for passive tumor immunotherapy is commented upon.

INTRODUCTION

The concept of transferring tumor-sensitized cells to a tumor bearer as a mode of immunotherapy has had appeal ever since the studies of Mitchison (15), Klein *et al.* (13), Winn (18), and others (2, 3, 5, 6, 11, 14, 16) emphasized the importance of the cellular component of the immune system in the host response to tumor. Despite extensive animal and human investigation [recently reviewed by Rosenberg and Terry (17)] directed toward ascertaining the efficacy of such therapy, little has been described concerning events which take place in recipients of such cells or the mechanism(s) responsible for inhibition of tumor growth following their administration. Whether the transferred cells are capable of eliciting a direct cytotoxic effect against tumor cells independent of host immune competence or whether host factors are involved has not been completely elucidated. It is likely that there will be insight into how best to use passive cellular immunotherapy (adoptive immunotherapy) only when there is an understanding of the mechanism(s) whereby such cells are effective. Consequently, since little consideration has been given to the response of a recipient to transferred cells, a series of investigations has been carried out by us to determine the effects of those cells on their recipients.

In previously reported studies (7, 10), we observed that transferal of RLNC,[3] nonregional LNC, or SPC from tumor-bearing mice to normal syngeneic recipients resulted in the production in the latter of LNC, SPC, and CMA which mediated specific *in vitro* cytolysis of immunizing tumor target cells. The transferal of myeloid cells, granulocytes, and CMA from tumor-bearing mice also produced cytotoxic cells. A reduction in cytotoxicity resulted following pretreatment of target tumor cells with serum derived from normal mice who had received either SPC or RLNC from tumor-bearing animals 7 days previously (10). The findings were considered to indicate that "information" had been transferred to normal animals that had never been exposed to a tumor, which resulted in their production of tumor-specific cytotoxic cells as well as serum inhibitory factor.

The purpose of this report is to present results obtained from investigations in which xenogeneic tumor-sensitized cells were transferred to normal recipients. Such cells were evaluated since they could have greater relevance to the use of passive immunotherapy than do syngeneic or even allogenic cells.

MATERIALS AND METHODS

Animals. Female C3HeB/FeJ mice, 8 to 12 weeks old, and female Fischer 344 Mai f rats, 8 to 12 weeks old, were used. All animals were housed in individual cages and fed laboratory chow and water *ad libitum*.

Tumors. A spontaneous mammary carcinoma arising in a

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[3] The abbreviations used are: RLNC, regional lymph node cells; LNC, lymph node cells; SPC, spleen cells; CMA, macrophages cultured from bone marrow; MC, methylcholanthrene.

female C3H/HeJ and carried in C3HeB female mice was used. This tumor, designated as the C3H mammary tumor, was used in all experiments. In experiments evaluating specificity, 3 additional mouse tumors were used: a mammary carcinoma produced by gastric instillation of MC (MC mammary); a sarcoma produced by s.c. injection of MC (induced fibrosarcoma); and a sarcoma spontaneously occurring in a C3HeB mouse (spontaneous fibrosarcoma). All tumors were transferred by the s.c. injection of 2×10^5 viable tumor cells in 0.1 ml Medium 199 in the left hind leg at the ankle. They were used when they are approximately 10 mm in diameter.

Sensitization of Rats. A tumor brei was prepared in Medium 199, and 0.25 ml was injected s.c. in each of the 4 extremities below the popliteal or epitrochlear nodes. Each rat received 300 to 500 mg of tumor. Control rats were given injections of the same amount of normal C3HeB kidney, spleen, or Medium 199 alone. Three injections were given at 7-day intervals, and the cells used for transfer were removed 7 days after the last injection. Only the lymph nodes regional to the 4 injection sites were used for transfer.

Cell Preparation and Testing. The methods of cell preparation for transfer and cytotoxicity assay, the production of macrophages by culture of bone marrow cells, microcytotoxicity procedure, and serum inhibition were the same as those used in the transfer of syngeneic cells (10). They have all been previously described in detail (4, 8, 9, 12).

Transfer of Cells. The cell suspensions were diluted in Roswell Park Memorial Institute Medium 1640 to contain 5×10^7 cells/ml. Each mouse received 5×10^6 cells via the tail vein. Mice were sacrificed 7 days after cell transfer. An aliquot of each rat cell suspension was resuspended in Earle's medium for cytotoxicity assay.

Statistical. Analysis was performed by the Mann-Whitney *U* test.

Experimental Design. To clarify the sequence of procedures used, the experimental design is depicted in Chart 1.

RESULTS

Cytotoxicity of Rat Cells following Sensitization with C3HeB Mouse Tissue. Sensitization of rats with normal or malignant cells from C3HeB mice resulted in rat LNC which displayed cytotoxicity to C3H mammary tumor target cells. The degree of cytotoxicity was similar when rats were sensitized with normal mouse tissue, C3H mammary tumor, or other tumor types. Almost no cytotoxicity was displayed by unsensitized rat LNC (Table 1). The SPC from sensitized rats also demonstrated cytotoxicity, but to a lesser degree than did the LNC. As when LNC were evaluated, the cytotoxicity of SPC was similar whether normal or tumor tissue had been used in the sensitization, and SPC from nonsensitized rats displayed minimal cytotoxicity.

Cytotoxicity of CMA of Normal Mice, Recipients of Sensitized Rat LNC. Transfer to mice of nonsensitized rat LNC resulted in mouse CMA which displayed little or no cytotoxicity when tested against each of the 3 tumor targets used (Table 2, Column A). When nontumorous mouse tissue (Table 2, Column B) was used for rat sensitization, the CMA of mice which were recipients of rat cells displayed little cytotoxicity to any of the tumor cell targets, although that quality was greater when kidney was used for sensitization than when spleen was used. In all investigations in which mouse tumors were used for rat sensitization (Table 2, Columns C, D, E, and F), except those in which the MC-induced fibrosarcoma was used (Table 2, Column D), the degree of cytotoxicity against the tumor target was consistently greater ($p < 0.001$) than when nontumorous tissue was used. Maximal cytotoxicity was demonstrated when the sensitizing and target tumors were the same. This was significantly ($P \leq 0.001$) greater than that displayed when the 2 were different.

In contrast to evidence of cross-reactivity between C3H mammary carcinoma, MC-induced mammary carcinoma, and spontaneous fibrosarcoma, there was no evidence for this when the MC-induced fibrosarcoma was the sensitizing tumor. With the latter tumor, the cytotoxicity was no greater than that resulting from sensitization with nontumorous mouse tissue ($p \geq 0.05$). The cytotoxicity of the MC-induced fibrosarcoma cells was not evaluated against itself because of inability to obtain satisfactory cultures of that tumor.

Cytotoxicity of LNC and SPC from Normal Mice or Recipients of Sensitized Rat LNC or SPC (Table 3). Transfer to mice of nonsensitized rat LNC or SPC resulted in mouse LNC and SPC which displayed little cytotoxicity when tested against the immunizing tumor (C3H mammary). When nontumorous mouse tissue (spleen cells) was used for sensitization of the rat, the LNC or SPC from recipient normal mice displayed only a slightly greater degree of cytotoxicity. The use of C3H tumor tissue to sensitize the rat resulted in a much higher degree of cytotoxicity in the mouse LNC and SPC ($p < 0.001$) than when nontumorous tissue was used for rat sensitization in all but one investi-

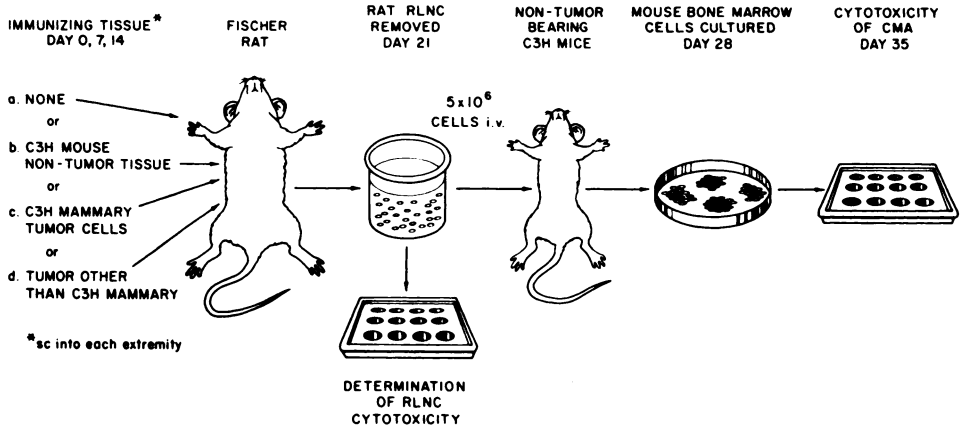

Chart 1. Experimental design in xenogeneic studies.

gation, Experiment 3 ($p = 0.07$).

Increased Cellularity in Lymph Node, Spleen, and Bone Marrow of Recipients of Various Cytotoxic Cells (Table 4). The transfer of LNC or SPC derived from normal nonsensitized rats resulted in increased cellularity of the lymph node, spleen, and bone marrow of normal recipient mice. When the transferred rat cells (RLNC or SPC) were derived from rats sensitized with normal mouse spleen or either mouse tumor, no significant increase in cellularity of mouse lymph node, spleen, or bone marrow beyond that observed following transfer of tissue from nonsensitized rats was observed.

Serum Inhibition of Cellular Cytotoxicity (Table 5). When normal mouse serum was used in the cytotoxicity testing (Group 1), 45.2% tumor cell destruction occurred. Serum from normal mouse recipients of nonsensitized rat LNC (Group 2) displayed no inhibiton of cellular cytotoxicity. When mice were recipients of LNC from rats sensitized to the C3H target tumor (Group 4), their sera were capable of inhibiting *in vitro* cytotoxicity to the same degree as sera from mice bearing the same tumor (Group 3). Sera from mice which were recipients of LNC from rats sensitized with C3H nontumorous tissue (spleen; Group 6) or with a tumor differing from the target (Group 7) resulted in an inhibition, equivalent in both groups, which was approximately one-half that resulted following immunization with the target tumor. When the SPC from rats immunized with C3H tumor (Group 5) were transferred to normal mice, the inhibition by serum from such mice was almost identical to that occurring when LNC were used.

Table 1

Cytotoxicity of rat cells following sensitization with C3H tissue

	% of tumor cell destruction[a]	
Type of C3H tissue	LNC	SPC
None	2.7 ± 0.48[b] (7)[c]	2.7 ± 0.40 (3)
Normal	33.6 ± 3.11 (7)	14.2 ± 4.37 (3)
C3H mammary tumor	35.2 ± 5.02 (7)	16.4 ± 1.37 (3)
MC mammary tumor	26.9 (2)	ND[d]
Spontaneous sarcoma	41.1 (1)	ND
Induced sarcoma	31.4 ± 2.87 (3)	ND

[a] C3H mammary carcinoma tumor cells served as target.
[b] Mean ± S.E.
[c] Number in parentheses, number of experiments.
[d] ND, not done.

DISCUSSION

The reported findings indicate that rat cells (LNC or SPC) sensitized to mouse tumor, when transferred to non-tumor-bearing normal mice, resulted in the development of cytotoxicity in recipient CMA, LNC, or SPC. These results are similar to those previously observed when syngeneic cells had been transferred (10). The cytotoxicity was not due to the presence of xenogeneic cells *per se*, since transfer of unsensitized rat cells or those sensitized to normal mouse tissue resulted in a display of little cytotoxicity by recipient cells. Rat cells sensitized to normal mouse tissue were, however, at the time of their transfer, capable of lysing tumor target cells. These findings

Table 2

Cytotoxicity of CMA from normal mouse recipients of sensitized rat LNC

		% of tumor cell destruction for the following rat-sensitizing tissues					
				Mouse tumor			
Experiment	Target tumor cell	None (A)	Mouse nontumorous[a] (B)	C3H mammary (C)	MC-induced fibrosarcoma (D)	MC-induced mammary (E)	Spontaneous fibrosarcoma (F)
1	C3H mammary	0.0	12.9	43.5			
2	C3H mammary	2.0	12.2	49.5		30.6	
3	C3H mammary	0.0	4.1	45.2		24.0	
4	C3H mammary	1.4	1.0	39.0	1.4		19.3
5	C3H mammary	3.5	4.1	26.1	8.2		
6	C3H mammary	2.9	5.5	35.7	6.9		
7	C3H mammary	0.6	2.6	27.9	8.8		
8	MC mammary	2.9	12.3	19.2		51.3	
9	MC mammary	1.0	6.1	23.8		44.3	
10	Spontaneous fibrosarcoma	1.0	0.0	19.9	7.9		35.9

[a] Experiments 1, 2, and 8, kidney; other experiments, SPC.

Table 3

Cytotoxicity of LNC and SPC from normal mouse recipients of sensitized rat LNC or SPC

				% of tumor cell destruction for the following rat-sensitizing tissues		
Experiment	Target tumor cell	Rat cell transferred	Recipient mouse cell tested	None (A)	Mouse nontumorous[a] (B)	C3H mammary tumor (C)
1	C3H mammary	LNC	LNC	5.5	10.3	26.1
2	C3H mammary	LNC	LNC	1.4	3.5	35.7
3	C3H mammary	SPC	LNC	5.5	11.1	17.9
4	C3H mammary	SPC	LNC	0.8	4.1	21.4
5	C3H mammary	LNC	SPC	3.5	4.9	22.6
6	C3H mammary	LNC	SPC	0.8	0.8	18.5
7	C3H mammary	SPC	SPC	2.1	4.1	21.9

[a] SPC from normal mice.

Table 4

Effect of cell transfer on the number of cells in lymph node, spleen, and bone marrow of recipients

Mouse tissue used for rat sensitization	Rat cells transferred	No. of determinations	No. of cells ($\times 10^6$) in recipient[a]		
			Lymph node	Spleen	Bone marrow (femur)
None	None	6	3.5 ± 0.11[b]	81 ± 4	3.5 ± 0.22
None	LNC	6	6.1 ± 0.65	158 ± 8	6.3 ± 0.24
None	SPC	3	8.5 ± 1.19	146 ± 32	5.3 ± 0.20
C3H spleen	RLNC	5	7.7 ± 0.18	179 ± 2	6.3 ± 0.21
C3H spleen	SPC	3	8.9 ± 0.54	170 ± 39	5.3 ± 0.80
C3H mammary tumor	RLNC	6	6.5 ± 0.36	109 ± 5	6.2 ± 0.14
C3H mammary tumor	SPC	3	7.7 ± 0.99	126 ± 22	5.9 ± 0.22
MC-induced sarcoma	RLNC	6	6.4 ± 0.62	113 ± 5	5.4 ± 0.28
MC-induced sarcoma	SPC	3	7.1 ± 0.58	179 ± 49	4.9 ± 0.83

[a] Seven days after transfer of 5×10^6 cells.
[b] Mean ± S.E.

indicate that rat cells possessing anti-mouse (H-2) properties, when transferred to a mouse, behave differently *vis-à-vis* that host than do similar cells sensitized to tumor as well as mouse. The former failed to "program" normal mouse cells, so that they or their progeny (CMA) displayed the cytotoxicty toward mouse tumor cells which occurred following transfer of the latter.

The dispersion and dilution of the transferred cells makes it unlikely that the cytotoxicity being demonstrated in the recipient was in those cells. Additional evidence negating that possibility comes from the finding indicating that rat cells sensitized to H-2 antigen alone or to H-2 plus tumor antigen, while equally cytotoxic at the time of their transfer, resulted in different degrees of cytotoxicity in the recipient. If the cytotoxicity being determined in recipients was only in transferred cells, the findings should have been quantitatively similar when either cell type was used.

In the previously reported studies with sensitized syngeneic cells (10), it was found that their treatment with mitomycin C or radiation before transfer did not prevent the development of cytotoxicity. Thus, replication of the transferred cells was not essential, and, consequently, the cytotoxicity identified was not in the progeny of the transferred cells. Disruption by freeze-thawing did completely abolish their ability to induce cytotoxicity in host cells, indicating the need for intact cells to accomplish this. Evidence was obtained in the syngeneic studies which indicated that transferred tumor antigen was not responsible for inducing cytotoxicity in recipient cells. Treatment of those cells with trypsin or pronase removed surface membrane, as demonstrated by immunofluorescence. When transferred, they produced cytotoxicity in recipients to the same degree as did cells which were not treated with enzyme prior to transfer. The experimental conditions in the present studies are entirely similar to those using syngeneic cells. Consequently, there is reason to anticipate that the various findings obtained with syngeneic cells should correspond if xenogeneic cells were similarly evaluated.

In the syngeneic system, a high degree of specific cytotoxicity to the immunizing tumor was observed when the spontaneous (C3H) and induced (MC) mammary tumors were evaluated. The present studies likewise demonstrated that the greatest cytotoxicity occurred when the immunizing and target tumor cells were the same. There was evidence of cross-reactivity, suggesting common antigenicity between the tumors.

As in the syngeneic system, tumor-sensitized xenogeneic cell transfer resulted in increased cellularity of nodes, spleen, and bone marrow. Whereas non-tumor-sensitized syngeneic cells produced no change in cellularity following transfer, an increase was seen when unsensitized or mouse spleen-sensitized xenogeneic cells were used. Such findings suggest that all of the types of transferred xenogeneic cells, following their lodgement, equivalently recruited host cells, either directly or indirectly through host cellular responses. The behavior of the recruited cells, *i.e.*, their cytotoxicity, was, however, entirely dependent upon the use of tumor for sensitization of the donor cells.

Table 5

Serum inhibitory factor in normal recipients (mice) of xenogeneic sensitized lymphoid cells

C3H tumor cells served as target for cytotoxicity testing. Effector cell (RLNC) from mice with 21-day C3H tumor.

Group	Rat-sensitizing tissue	Rat cells transferred to normal mice	Donor of mouse serum	% of tumor cell destruction
1	None	None	Normal	45.2
2	None	LNC	Normal	45.9
3	None	None	C3H Tumor-bearing	25.5 (44.4)[a]
4	C3H tumor	LNC	Normal	27.5 (40.1)
5	C3H tumor	SPC	Normal	28.1 (38.8)
6	C3H nontumorous (spleen)	LNC	Normal	35.7 (22.2)
7	MC fibrosarcoma	LNC	Normal	36.9 (19.6)

[a] Number in parentheses, percentage of decrease in cytotoxicity compared to Group 2.

No evidence of a graft *versus* host or a hypersensitivity reaction was observed in the animals used in these studies or in other normal mice given 3 weekly injections of 10^8 to 10^{10} tumor-sensitized rat SPC. None of the mice lost weight, appeared sick, or died during a 3-month period of observation. When planning the use of allogeneic or xenogeneic cells for adoptive immunotherapy, consideration has frequently been given to attenuating the host immune response so as to permit a more protracted survival of the transferred cells, since it is generally held that it is those cells which possess the capability of directly destroying tumor. Less attention has been given to the possibility that they could exert an antitumor effect via a host-mediated response (1). The present findings provide no information supporting or denying the former mechanism, *i.e.*, that transferred cells act directly on tumor. They do, however, indicate that the host is involved. Consequently, the adminis-

tration of immunosuppressive agents to permit prolongation or survival of allogeneic or xenogeneic cells may be inappropriate, since those agents could obtund a favorable host response. Moreover, the use of chemotherapy or radiation in conjunction with such cells in a combined-modality approach to cancer therapy may be critically affected by the timing of administration of the modalities.

In the syngeneic studies, sera from normal cell recipients were capable of inhibiting the cytotoxicity demonstrated by lymphoid cells derived either from recipients or from tumor-bearing animals. In the present studies, similar findings were obtained. Sera from mice which were recipients of LNC from rats sensitized to C3H target tumor inhibited cytotoxicity to the same extent as did sera from mice bearing the C3H tumor. Even though such inhibition in this system was highly nonspecific, the concomitant development of both serum inhibitory factor and cellular cytotoxicity could account for difficulty in producing tumor growth inhibition when using adoptively transferred cells.

The mechanism(s) whereby transferred cells initiate cytotoxicity can at present, only be speculative. Whether direct contact between donor and recipient cells is required, whether "transfer factor" is elaborated by the administered cells or there is transferal of "immune RNA," remains conjectural. Whatever the explanation, it would seem that in the xenogeneic system, as in the syngeneic, information has been transferred to normal animals never exposed to tumor, resulting in cells which possess a high degree of tumor-specific cytotoxicity. Of particular importance in these studies was the finding that only when rat cells were sensitized to tumor did their transfer result in tumor cytotoxicity in the cells of normal mouse recipients. The use of normal mouse tissue possessing H-2 but devoid of tumor antigen for sensitization of xenogeneic cells failed to result in recipient cells which were tumor cytotoxic.

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