

## REJECTION OF TUMOR ALLOGRAFTS BY MOUSE SPLEEN CELLS SENSITIZED IN VITRO\*

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The rejection of solid allografts is mediated primarily by sensitized lymphoid cells rather than by humoral antibodies (1). However, the cellular processes involved in induction of lymphocyte sensitization and in lymphocyte-mediated destruction of allografted cells are not well characterized. Although much is known about the genetics of histocompatibility reactions, particularly those of the mouse (2), experimental systems *in vivo* do not provide means of studying these problems directly. Hence, numerous systems have been developed in an attempt to study allograft reactions *in vitro* (3). Unfortunately, most of these *in vitro* systems are limited to studying the cytotoxic effects against target cells of lymphocytes previously sensitized *in vivo* (3-8). Such systems thus do not provide a way of directly studying the induction of sensitization which initiates the allograft reaction.

In order to investigate some of these problems, we modified a system of cellular immunity (9-12) to promote the sensitization of mouse spleen cells against allogeneic antigens *in vitro* (13). Achievement of functional allosensitization *in vitro* was assayed by testing the ability of the spleen cells to inhibit the growth of tumor allografts *in vivo*. The cytolytic effects of sensitized cells against allogeneic target fibroblasts *in vitro* was also tested.

### *Materials and Methods*

*Animals.*—We employed mice of the strains C57BL/6J and C3H/ebJ or C3H/ebw. The latter was developed at the Experimental Animal Center of the Weizmann Institute. These mice are free of the typical C3H tail lesions. *In vivo* studies were performed on 8-10 wk old mice of both sexes. We used inbred Lewis (AgB-1) or Wistar (AgB-unknown) rat strains.

*Fibroblast Cultures.*—Primary monolayers of fibroblasts were derived from 16 to 17-day old embryos. The fibroblast cultures were prepared and maintained as previously described (9-12). The fibroblasts were transferred to sensitization cultures ( $3 \times 10^6$  fibroblasts in 60

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mm plastic dishes [Falcon Plastics, Los Angeles, Calif.] and target cultures ( $0.7 \times 10^6$  fibroblasts in 35 mm plastic dishes) after X-irradiation (2000 R). Target fibroblasts were labeled 4–5 days before use with radioactive chromium by incubation with  $^{51}\text{Cr}$  as  $\text{Na}_2\text{CrO}_4$  (Radiochemical Center, Amersham, England) at a concentration of 2  $\mu\text{Ci}$  per target plate (10–12).

*Sensitization In Vitro.*—Male mice, 3 months of age, were used as donors of lymphoid cells. The animals were sacrificed, and spleens, lymph nodes, or thymuses were removed into sterile, cold phosphate-buffered saline (PBS),<sup>1</sup> pH 7.2. The cells were freed into cold PBS by expressing the organs through a fine wire mesh using a syringe plunger. Bone marrow cells were collected by washing femur marrow spaces with cold PBS. Suspensions of mouse cells were washed in cold PBS, centrifuged (600 g, 7 min), counted in a hemocytometer, and resuspended in Dulbecco's modification of Eagle's medium (EM) with 20% fetal calf serum (FCS) (Grand Island Biological Co., Berkeley, Calif.). Batches of serum were used after they were shown to have no detrimental effects on lymphoid cell cultures. Unless stated otherwise,  $35 \times 10^6$  nucleated spleen cells in 4 ml of EM + FCS were seeded on each sensitizing fibroblast monolayer. In some studies hydrocortisone sodium succinate (Organon, Inc., West Orange, N.J.) or prednisolone sodium tetrahydrophthalate (Ciba Corp. Summit, N.J.), 1  $\mu\text{g}/\text{ml}$ , were added to the culture medium in order to promote sensitization (12). Sensitization cultures were incubated at 37°C in 10%  $\text{CO}_2$  in moist air. After the 3rd day of culture, 3 ml of medium was withdrawn and replaced by fresh EM + FCS. Glucocorticoids were not added to the fresh medium at this time. At the end of the 5th day the lymphoid cells were collected from the sensitizing fibroblast cultures by repeated pipetting of the culture medium, and were resuspended in cold PBS. The cells were examined and counted. Fibroblasts were distinguishable by their characteristic appearance. About  $1\text{--}2 \times 10^6$  nucleated spleen or lymph node cells were usually recovered from each sensitization culture which included glucocorticoids. About 60–80% of the recovered cells appeared to be large blast-like cells. In the absence of added glucocorticoids, about  $5\text{--}6 \times 10^6$  cells, 15–45% large, were usually recovered from each culture. Cultures of thymus cells usually yielded a much smaller number, and bone marrow cells a larger number of cells.

Rat lymph node cells were obtained as described previously (9–12) from rats weighing about 300 g. Rat lymphocytes were cultured with sensitizing fibroblasts in Dulbecco's modification of Eagle's medium with 20% horse serum (HS).

*Sensitization In Vivo.*—The 5 day sensitization schedule was duplicated in vivo by injecting mice with  $1 \times 10^6$  strain-specific tumor cells by the intraperitoneal route and removing the spleens after the 5th day. Suspensions of these in vivo-sensitized spleen cells were prepared by expressing the spleens through a fine wire mesh into cold PBS.

*Assay of Cytolytic Effects.*—Injury to target fibroblasts was measured by the  $^{51}\text{Cr}$  method developed by Berke and associates (10) with minor modifications (11, 12). Sensitized lymphoid cells were resuspended in EM + FCS (mouse) or EM + HS (rat), transferred in 1.5 ml to each of three  $^{51}\text{Cr}$ -labeled target fibroblast monolayers, and cultured for 20 hr. Cell-mediated damage to target fibroblasts was measured as the per cent of  $^{51}\text{Cr}$  label released into the medium, minus that measured in control cultures (usually 5–15%). Gamma radiation was measured in a well-type sodium iodide crystal scintillation counter (Packard Instrument Co. Inc., Downers Grove, Ill.). Each experimental value represents the mean of three target cultures.

*X-Irradiation.*—Mice were exposed to total body radiation of 550 R, using a Picker-Vanguard X-ray machine (250 kv, 15 ma). They were irradiated at a target distance of 50 cm (dose rate, 60 R/min) with 0.5 mm Cu and 1.0 mm Al added filtration.

<sup>1</sup> Abbreviations used in this paper: EM, Eagle's medium; FCS, fetal calf serum; HS, horse serum; PBS, phosphate-buffered saline.

*Tumors.*—The tumors used were fibrosarcomas produced by subcutaneous injections of 0.2 ml of 1.5% benzo(a)pyrene in sesame oil. Sarcoma H-1 was induced in a mouse of C3H strain, and sarcoma SBL5 in one of C57BL strain. These tumors were strain specific and showed progressive growth only in mice of their strain of origin (14). They could grow, however, in sublethally irradiated allogeneic recipients (15). Tumor T-1 was induced in a mouse of C3H strain and D-2 in a mouse of C57BL strain. These tumors were used in their second to fourth transfer generation and were also found to be strain specific. Suspensions of tumor cells were obtained by trypsinization of excised tumor tissue.

*Inhibition of Tumor Growth.*—In preliminary experiments it was found that a dose of  $1 \times 10^6$  sarcoma cells produced palpable tumors in allogeneic recipients when the mice were injected within 24 hr after irradiation. In addition, this dose of sarcoma cells was not great enough to obscure the inhibitory effect of sensitized lymphoid cells. Thus, in the in vivo system,  $1 \times 10^6$  sarcoma cells in 0.1 ml of PBS were mixed with 0.1 ml of PBS with or without lymphoid cells. The cell mixture was injected by intramuscular route into the right thigh of irradiated recipient mice. The mice were examined for palpable tumors at intervals, for periods of up to 30–40 days.

## RESULTS

*Inhibition of Tumor Allografts by Spleen Cells Sensitized In Vitro.*—The first series of experiments was aimed at testing whether mouse spleen cells sensitized in vitro will inhibit the growth of tumor allografts.

Table I shows the cumulative results of seven experiments in which the growth of allogeneic tumor cells was tested after injection of tumor cells together with mouse spleen cells allosensitized in vitro. Control mice were injected with tumor cells alone, or with a mixture of tumor cells and unsensitized mouse spleen cells or spleen cells sensitized in vitro against rat fibroblasts. It was found that only 4% ( $\frac{5}{124}$ ) of mice injected with tumor cells together with an equal number of specifically allosensitized spleen cells developed tumors. The allogeneic tumors were ultimately rejected in most experiments after the recipient mice apparently recovered from the immunosuppressive effects of irradiation (Fig. 1). However, the high degree of sensitization induced in vitro was demonstrated by the ability of allosensitized spleen cells to inhibit even tumors which were potentially lethal. Fig. 2 shows the time course of experiment 1 (Table I). In this experiment, in contrast to the others, all six control mice died as a result of massive tumor growth. Five of the six mice died within 18 days and one died 40 days after injection of sarcoma cells. Nevertheless, tumor growth was completely inhibited in 9, and only a transient tumor appeared in 1, of the 10 mice in the experimental group.

*Inhibition of Tumor Allografts by Spleen Cells Sensitized In Vivo.*—The above findings prompted us to compare the efficiency of this tumor inhibition to that produced by spleen cells sensitized in vivo. Table II demonstrates the effects of injecting irradiated recipient mice with  $1 \times 10^6$  D-2, C57BL strain tumor cells, together with various numbers of unsensitized spleen cells, or spleen cells derived from mice allosensitized in vivo or in vitro. It can be seen that  $1 \times 10^6$  spleen cells allosensitized in vivo did not prevent the growth of  $1 \times 10^6$  allo-

genic tumor cells. However, increasing the number of these spleen cells to  $5 \times 10^6$  (group 3) or  $10^7$  (group 4) was associated with inhibition of tumor growth, similar to that produced by  $1 \times 10^6$  spleen cells allosensitized in vitro

TABLE I  
*Inhibition of Tumor Allografts by Spleen Cells Sensitized In Vitro\**

Exp.No.	X-irradiated recipient mice	Tumors (strain of origin)	Control spleen cells		Allosensitized spleen cells	
			Type	Incidence of palpable tumors	Type	Incidence of palpable tumors
1	C57BL	H-1 (C3H)	None	6/6	C57BL anti-C3H	1/10
2	C57BL	H-1 (C3H)	C57BL anti-Lewis rat	6/10	C57BL anti-C3H	0/15
3	C57BL	H-1 (C3H)	C57BL anti-Wistar rat	7/8	C57BL anti-C3H	0/23
4	C3H	SBL-5 (C57BL)	Unsensitized C3H	6/8	C3H anti-C57BL	2/11
			Unsensitized C57BL	5/8		
5	C57BL	T-1 (C3H)	C57BL anti-Lewis rat	7/10	C57BL anti-C3H	0/8
			None	5/8	C57BL† anti-C3H	0/10
6	C3H	D-2(C57BL)	None	28/28	C3H anti-C57BL	0/35
7	C57BL	H-1 (C3H)	C3H anti-C57BL	12/12	C57BL anti-C3H	2/12
		Total incidence of palpable tumors		82/98		5/124
		Per cent		83		4
		$P < 0.001$				

\*  $35 \times 10^6$  nucleated spleen cells were sensitized in tissue culture against fibroblasts in the presence of  $1 \mu\text{g/ml}$  of hydrocortisone. In experiment 5, the spleen cells marked (†) were sensitized without added hydrocortisone.  $1 \times 10^6$  tumor cells alone or mixed with  $1 \times 10^6$  spleen cells were injected into X-irradiated recipient mice.

(Tables I and II). Unsensitized control spleen cells did not prevent the development of tumor even at doses of  $10^7$  cells (group 5). These findings suggest that populations of spleen cells sensitized in vitro may be several times more active than populations of spleen cells obtained after sensitization in vivo.

*Cytolytic Effects of Rat and Mouse Lymphoid Cells In Vitro.*—It has been shown that lymph node cells of rats can be both sensitized against, and cause injury to

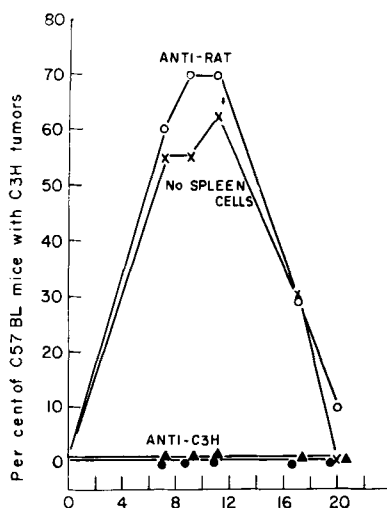


FIG. 1. Inhibition of C3H tumors by C57BL spleen cells sensitized in vitro. C57BL spleen cells were sensitized against fibroblasts of a Lewis rat or C3H mouse with (●—●), or without 1  $\mu$ g/ml of hydrocortisone (▲—▲), and were injected into recipient mice together with tumor cells (Table I, exp. 5).

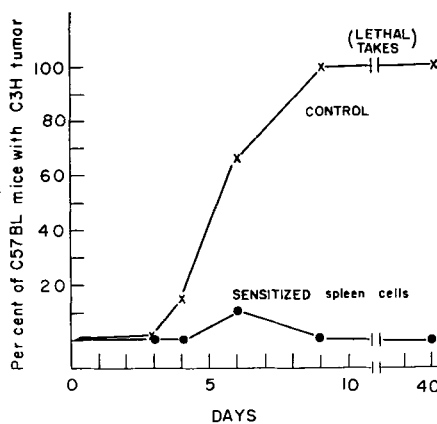


FIG. 2. Prevention of lethal takes of C3H tumors by C57BL spleen cells sensitized against C3H fibroblasts in vitro (Table I, exp. 1).

allogeneic rat (11-13) or mouse (9-11) fibroblasts in vitro. We, therefore, attempted to assay the cytolytic capacity of allosensitized mouse lymphocytes against allogeneic target fibroblasts in vitro. Table III shows the results of an experiment in which lymph node cells were obtained from Lewis rats and

C57BL mice, and  $20 \times 10^6$  were sensitized against C3H mouse or Wistar rat fibroblasts in vitro. It was found that  $3 \times 10^6$  allo- or xenosensitized Lewis

TABLE II  
*Effect on Allograft Tumor Growth Produced by Spleen Cells Sensitized In Vitro, as Compared with Cells of Immunized Mice or with Untreated Spleen Cells\**

Group No.	Strain origin of		Sensitization route	Tumor growth †				
	Spleens tested	Sensitizing cells		Experimental				Control §
				$2 \times 10^7$	$10^7$	$5 \times 10^6$	$10^6$	
1	C3H	C57BL	In vitro	N.T.	N.T.	N.T.	0/35	28/28
2	C57BL	C57BL	In vitro	N.T.	N.T.	N.T.	35/35	
3	C3H	C57BL	In vivo, i.p. injection of cells	0/10	0/10	0/10	4/9	14/16
4	C3H	C57BL	In vivo, s.c. injection of cells	0/9	0/10	3/10	4/10	
5	C3H	—	—	1/9	6/10	8/9	10/10	19/19
6	C57BL	—	—	9/9	10/10	10/10	10/10	

N.T., not tested.

\* D-2 fibrosarcoma of C57BL/6 origin was tested in C3H/ebw irradiated mice.

† Mice were inoculated with a mixture of  $10^6$  tumor cells with different doses of test cells.

§ Mice were inoculated with  $10^6$  tumor cells only.

|| Included in Table I, exp. 6.

TABLE III  
*Cytolytic Effects of Rat and Mouse Lymph Node Cells Sensitized In Vitro*

Sensitization phase		Cytolytic effector phase*	
Lymph node cells ( $20 \times 10^6$ )	Sensitizing fibroblasts	$^{51}\text{Cr}$ released from target fibroblasts	
		C3H	Wistar
		(%)	(%)
Lewis rat	Wistar rat	7	32
Lewis rat	C3H mouse	38	5
C57BL mouse	C3H mouse	0	0

\*  $3 \times 10^6$  sensitized lymph node cells per target culture.

lymphocytes produced immunospecific release of  $^{51}\text{Cr}$  from the target cells against which they had been sensitized. In contrast, the C57BL mouse lymphocytes were unable to cause either specific or nonspecific release of  $^{51}\text{Cr}$  from target cells.

Resistance of C3H target fibroblasts to lysis could not explain the apparent

failure of mouse lymph node cells to produce cytolytic release of  $^{51}\text{Cr}$ , since these target fibroblasts were susceptible to injury by sensitized rat lymphocytes. It was possible, however, that the mouse lymph node cells were not adequately sensitized. We therefore sensitized C57BL spleen cells against C3H or Lewis rat fibroblasts, with or without the presence of hydrocortisone in the sensitizing cultures. Table IV shows the results of assaying these cells for cytolytic effects against specific target fibroblasts *in vitro*, and for inhibition of growth of C3H tumor cells *in vivo*. As demonstrated in the experiments cited

TABLE IV  
*Effects of Sensitized C57BL Mouse Spleen Cells on Target Fibroblasts In Vitro and on the Growth of Tumor Allografts In Vivo*

Sensitization phase <i>in vitro</i> *		Effector phase		
Sensitizing fibroblasts	Hydrocortisone	<i>In vitro</i> †		<i>In vivo</i> ‡
		$^{51}\text{Cr}$ released from target fibroblasts		Incidence of palpable C3H tumors (T-1)
		(%) C3H	(%) Lewis	
C3H	+	4	N.T.	0/10
	-	0	N.T.	0/7
Lewis rat	+	N.T.	0	N.T.
	-	N.T.	0	7/10
None	-	0	0	5/8

N.T., not tested.

\*  $45 \times 10^6$  nucleated spleen cells were obtained from C57BL mice and sensitized against C3H mouse or Lewis rat fibroblasts with or without  $1 \mu\text{g/ml}$  of hydrocortisone in the culture medium.

†  $3.5 \times 10^6$  sensitized spleen cells per target culture.

‡ Included in Table I, exp. 5.

above, the allosensitized C57BL spleen cells completely inhibited the growth of C3H tumors *in vivo*. This indicates that functional allosensitization was induced. Nevertheless, the sensitized mouse spleen cells had no demonstrable cytolytic effects on either C3H mouse or Lewis rat target fibroblasts in cell culture.

These findings suggested that cytotoxic effector cells may have been deficient in sensitization cultures derived from mouse spleens as well as in those derived from lymph nodes. A recent analysis of the cytolytic effects of rat lymphoid cells against target fibroblasts (16) indicated that the reaction is based upon cooperation between two types of lymphocytes, one of thymus and the other of nonthymus origin. We therefore carried out six experiments in which cells for *in vitro* sensitization were obtained from thymus or bone marrow, as

well as from spleen or lymph nodes. However, allosensitization of mouse lymphoid cells derived from any of these sources, alone or in various combinations, did not lead to cells capable of causing significant lysis of target fibroblasts in vitro. Table V shows the results of one of these studies.

*Inhibition of Lymphocyte Sensitization by Anti-Fibroblast Antibodies.*—The immunospecificity of the induction of sensitization of rat lymphocytes against mouse fibroblast antigens was studied previously (17). It was found that sensitization of lymphocytes in vitro could be inhibited by preincubating the sensitizing fibroblasts with rat anti-mouse antiserum. The magnitude of inhibition

TABLE V  
*In Vitro Cytolytic Effect of C57BL Mouse Lymphoid Cells Sensitized In Vitro against C3H Fibroblasts*

Sensitization phase*	Cytolytic effector phase†	
	<sup>51</sup> Cr released from target fibroblasts	
	C3H	Lewis
Type of lymphoid cells	(%)	(%)
Spleen	1	3
Bone marrow	4	3
Lymph node	4	2
Thymus	1	4
Bone marrow + thymus + spleen	4	3

\*  $30 \times 10^6$  C57BL lymphoid cells, or mixtures of  $10 \times 10^6$  of each of three types, were sensitized in vitro against C3H fibroblasts.

†  $4 \times 10^6$  cells per target culture.

of sensitization appeared to correlate with the concentration of antibodies binding to the sensitizing fibroblasts. It was concluded, therefore, that an immunospecific interaction between rat lymphocytes and mouse fibroblast antigens was necessary for the induction of lymphocyte sensitization. Hence, it would be expected that preincubation of sensitizing mouse fibroblasts with antisera to alloantigens also would inhibit the in vitro sensitization of allogeneic mouse spleen cells. To test this, C3H sensitizing fibroblasts were preincubated with C57BL anti-C3H antiserum or with normal serum. C57BL spleen cells were then added to the sensitizing cultures. C3H tumor cells were mixed with these sensitized spleen cells or with serum alone and injected into recipient irradiated C57BL mice. The results are shown in Table VI. It was found that 30% ( $\frac{3}{10}$ ) of the mice receiving tumor cells in normal serum developed tumors. C3H tumor cells injected together with C57BL anti-C3H antiserum produced tumors in 78% ( $\frac{7}{9}$ ) of the mice. The augmented growth of these C3H tumors suggests that rejection of tumors was not mediated by interaction with extracellular antibodies. Tumors did not develop (0/8) in mice which received tumor



cells together with C57BL spleen cells which were cultured with C3H fibroblasts in the presence of normal serum. This result is in accord with the low overall incidence of tumors (4%) in mice receiving allosensitized spleen cells, which was presented in Table I. This indicates that allosensitization was induced in the presence of normal serum. On the other hand, tumors developed in 25% ( $\frac{3}{12}$ ) of mice which were injected with C57BL spleen cells that had been cultured with C3H fibroblasts in the presence of the anti-C3H antiserum (Table VI). Although the numbers of mice used in this experiment were small, it is noteworthy that this incidence of tumors (25%) was about the same as the

TABLE VI  
*Effects of Antibodies on Sensitization of C57BL Spleen Cells and Growth of Tumors*

C3H tumor cells injected together with		Incidence of tumors in irradiated C57BL mice	
C57BL serum	Spleen cells*	Number	Per cent
Normal serum	—	3/10	30
Anti-C3H antiserum†	—	7/9	78
	Sensitized against C3H in the presence of:‡		
—	Normal serum	0/8	0
—	Anti-C3H serum	3/12	25

\*  $1 \times 10^6$  T-1 tumor cells were mixed with 0.1 ml of serum or with  $1 \times 10^6$  C57BL spleen cells.

† C57BL mice were injected intraperitoneally weekly for 8 wk with  $5 \times 10^7$  C3H spleen cells. Sera were collected 1 wk later.

‡ Sensitizing C3H fibroblasts were preincubated with 0.4 ml of normal or anti-C3H antisera diluted 1:2 in EM for 40 min. The sera were removed by aspiration before spleen cells were added for sensitization.

incidence of tumors in control mice injected with normal serum (30%). The failure of these spleen cells to inhibit tumor growth indicates that lymphocyte sensitization was abolished in the presence of antibodies to allogeneic fibroblast antigens.

#### DISCUSSION

This paper reports the induction of primary sensitization of mouse spleen cells against allogeneic fibroblasts *in vitro*. Achievement of functional allosensitization was indicated by the ability of these spleen cells to inhibit the growth of tumor allografts *in vivo*. Allo-antibodies binding to antigens of sensitizing fibroblasts appeared to block the allosensitization of spleen cells *in vitro*. This indicates, as suggested in earlier studies (17), that an immunospecific interaction between spleen cells and sensitizing fibroblasts is necessary to initiate the induction of sensitization. Injecting tumor cells together with preformed allo-

antibodies alone led to enhancement of tumor growth rather than to rejection. This suggests that inhibition of tumor growth in the presence of allosensitized spleen cells was not brought about by extracellular antibodies. Hence, the *in vitro* system appears to behave as a model in which cell-mediated allosensitization can be studied.

Hellström and Hellström (18) recently summarized the literature dealing with the "neutralization" of tumor allografts by lymphoid cells sensitized *in vivo* against transplantation antigens. They indicated that a ratio of 10:200 lymphoid cells per tumor cell is usually necessary in order to detect growth inhibition in X-irradiated recipient mice. We found that about  $5 \times 10^6$  *in vivo*-sensitized spleen cells were needed to inhibit  $1 \times 10^6$  tumor cells in irradiated recipients. In contrast to this cell ratio of 5:1, the spleen cells sensitized in cell culture were able to inhibit the growth of an equal number of tumor cells. It was found in a previous study (12) that sensitization in cell culture can promote the selection of specifically sensitized lymphocytes. Thus, the relative number of specifically active cells in a population of spleen cells after sensitization *in vitro* may be greater than that obtained after sensitization *in vivo*. Nevertheless, these highly sensitized populations of mouse spleen cells were not able to injure target fibroblasts *in vitro*.

The failure of the *in vitro*-sensitized mouse spleen cells to produce cytotoxic effects against target cells *in vitro* might be explained by a possible defect in their ability to recognize fibroblast antigens. This is unlikely, however, since sensitization was found to depend upon recognition of allo-antigens on the sensitizing fibroblasts *in vitro*. Recognition *in vivo* was demonstrated by the inhibition of tumor growth. Thus, the lack of *in vitro* cytotoxicity of these cells cannot be explained by a failure to recognize allo-antigens. This finding suggests, therefore, that recognition of antigen and cytotoxic effect are two distinguishable processes of sensitized lymphocytes.

We have recently analyzed the cytolytic effects of rat lymphocytes against target fibroblasts *in vitro* (11, 17). These studies provided more direct evidence that the effector response of sensitized lymphocytes can be divided into two major stages. The first stage appears to be activation of the cytolytic potential of sensitized lymphocytes. This appears to be brought about by immunospecific contact between lymphocytes and antigens. The second stage is the mediation of injury to the target cells. This stage does not seem to be immunospecific and antigen-activated lymphocytes were found to injure antigenically nonspecific fibroblasts, provided that close contact could be made (11, 17).

It is possible, therefore, that mouse spleen cells which have been sensitized *in vitro* lack the effector mechanism demonstrated in rat cells which is needed to injure target cells. This cytolytic mechanism may be activated by further differentiation of sensitized mouse lymphocytes *in vivo*. Alternatively, it may be speculated that injury may be mediated by an interaction between *in vitro*-

sensitized lymphocytes and "nonspecific" effector cells recruited from the recipient mice. Ax and associates (19) independently have developed an *in vitro* method for allosensitization of mouse lymphoid cells which is similar to ours. They report that these lymphoid cells are able to lyse allogeneic mastocytoma target cells which are highly susceptible to lysis (6). However, the failure of sensitized mouse cells to produce the cytolytic effects demonstrated by rat cells against the same target fibroblasts remains unexplained.

There is no direct evidence, on the other hand, that the process of cytotoxicity measured *in vitro* plays any role in the rejection of allografts *in vivo*. Thus, there might be no necessary functional relationship between the ability of lymphocytes to reject allografted cells or tissues and their ability to lyse target cells in cell culture. Sensitization is certainly not a prerequisite for lymphocyte-mediated injury to target cells. It has been shown that nonsensitized lymphocytes may be activated by a variety of nonimmune substances such as phytohemagglutinin to produce lysis of target cells (3, 20).

Attempts have been made to characterize lymphocyte recognition of alloantigens by studying cytotoxicity to target cells produced by sensitized lymphocytes (3). Brondz (8) has suggested that an immune lymphocyte can interact with a target cell only if there exists a suitably close fit between target alloantigens and a whole complex set of lymphocyte receptors. Mauel and associates (7) also interpreted their findings as suggesting a similar possibility. However, the results described here indicate that the conditions necessary for the expression of lymphocyte-mediated cytotoxicity may not be directly related to the process of antigen recognition itself. Hence, caution must be exercised in extrapolating the results of cytotoxic experiments to analyze the problem of lymphocyte recognition of antigen.

The *in vitro* system of allosensitization, on the other hand, provides a means of studying sensitization of lymphocytes which is triggered by recognition of antigens. In addition to inhibiting tumor allografts, spleen cells sensitized *in vitro* have been found to produce graft vs. host reactions in newborn animals (13). This model of cellular immunity, therefore, appears to be unique in that it is initiated *in vitro* but still is measurable *in vivo*.

We have found in recent studies that *in vitro*-sensitized spleen cells injected intraperitoneally reject allogeneic fibrosarcoma cells grafted separately into the thigh muscles or footpads of recipient mice. About  $15 \times 10^6$  *in vitro*-allosensitized spleen cells were able to transfer specific resistance to  $1 \times 10^6$  tumor cells. Similar numbers of nonsensitized spleen cells or spleen cells allosensitized *in vivo*, injected intraperitoneally, were unable to reject the tumor cells. These findings indicate that *in vitro*-allosensitized spleen cells need not be mixed with tumor cells locally to inhibit their growth *in vivo*. The ability of spleen cells to function systemically provides additional evidence of their state of allosensitization.

## SUMMARY

This paper reports a model system of cellular immunity in which allosensitization of mouse spleen cells is induced in vitro. Allosensitization was achieved by culturing spleen cells upon monolayers of allogeneic fibroblasts. The ability of the spleen cells to inhibit the growth of tumor allografts in vivo served as a functional assay of sensitization. We found that unsensitized spleen cells or spleen cells sensitized against unrelated fibroblast antigens had no inhibitory effect on the growth of allogeneic fibrosarcoma cells when they were injected together into irradiated recipients. In contrast, spleen cells which were specifically allosensitized in vitro were found to be highly effective in inhibiting the growth of an equal number of allogeneic tumor cells. Several times more spleen cells from mice sensitized in vivo were required to produce a similar immune effect. This confirms the findings of previous studies which indicate that sensitization in cell culture can promote the selection of specifically sensitized lymphocytes.

Preincubating sensitizing fibroblasts with allo-antisera blocked the allosensitization of spleen cells. This suggests that antibodies binding to fibroblasts may inhibit the induction of sensitization by competing with lymphocytes for antigenic sites.

Mouse spleen cells which were able to recognize and reject tumor allografts in vivo were unable to cause lysis of target fibroblasts in vitro. Such fibroblasts, however, were susceptible to lysis by rat lymphoid cells sensitized by a similar in vitro method. These findings indicate that the conditions required for lymphocyte-mediated lysis of target cells may not be directly related to the processes of antigen recognition and allograft rejection in vivo.

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