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Response of the Host Vascular System to Immunocompetent Lymphocytes: Effect of Preimmunization of Donor or Host Animals[1] (40514)

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We have previously reported that when immunocompetent lymphocytes are inoculated into the skin of irradiated, unimmunized mice, they evoke a complex vascular response pattern marked by the appearance of increased vessel tortuosity, changes in blood vessel dimensions, major alterations in capillary histology, and the generation of new blood vessels (1). We have given the reaction the name "Lymphocyte-Induced Angiogenesis" or LIA because of its characteristic neovascular component, although it is at once apparent that the total response to foreign lymphocytes includes many other facets collectively considered as comprising the inflammatory reaction and involving the entire spectrum of cells of the reticulo-endothelial system. The LIA assay involves the enumeration of new vascular branches appearing at a critical time after intradermal inoculation of foreign lymphocytes into immunologically inactive host animals.

The present experiments were designed to examine the effect of prior immunization of either donor or host animals on the nature and extent of the subsequently evoked angiogenesis response.

Materials and methods. Mice from our own colony included BALB/cAu, CBA/JAu, C_3H/HeAu, C57BL/6JAu, DBA/2JAu and Ha/ICR (H-2q), supplemented as needed by C57BL/6J and Ha/ICR mice purchased from ARS-Sprague-Dawley Company, Madison, WI. In early experiments male mice were used, while later experiments were carried out with female mice; no differences based on sex were noted.

Donor spleen cell suspensions were made by teasing and abrasion against a stainless steel screen immersed in 0.9% NaCl solution. Cells were washed twice in saline, viability was assessed by trypan blue exclusion and the cell concentration was then adjusted as required by experimental protocols. A drop of dilute trypan blue was added to the final suspension for subsequent identification of the injection site.

Intracutaneous inoculations were made with a 26-gauge needle which was introduced about 0.5 cm into the skin, as close to the epidermis as possible. 0.1 ml of the suspension was injected in all instances, and the progress of the injection was monitored by palpation. Animals routinely received 4 injections, two on each side, midlaterally in the thoracolumbar portion of the trunk. Regional differences were not observed in the LIA reaction in different inoculation sites (cf. 2).

To carry out the LIA assay mice were killed with ether after three days, and a midventral incision was made in the skin which was then separated from the underlying muscles. Superficial fascia were removed to expose the inoculation site. Scoring of all reaction areas was carried out under uniform magnification (7×) of a dissecting microscope. All extra blood vessels connected with the scar region and contrasting with the background vasculature were counted; these vessels were readily detectable because of their increased tortuosity and tendency to loop formation. Details of the method have been presented in an earlier publication which also includes photographs of the reaction (1).

Irradiation was carried out using 300 kvp, 20 mA X-rays with 1 mm Cu and 0.5 mm Al added filtration delivered from a GE maxitron unit operating at a dose rate of approximately 85R as measured in air by a Victoreen dosimeter.

Animals were immunized by injecting 6×10^7 spleen cells divided into 3 aliquots, one administered intraperitoneally, the others by intracutaneous injection in the dorsal skin. In general, animals were used 10–15 days after the day of immunization.

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Results. Donor preimmunization. A variety of strain combinations were used, involving both H-2 and M-locus disparity. Cells from animals immunized against an unrelated (cross-reactive?) strain were tested in parallel to establish the degree of specificity of observed effects of preimmunization. The results of experiments carried out with donor BALB/c cells are shown in Table I. Experiments involving other strain combinations (not shown) gave comparable results. As can be seen from these data, there was a specific measurable effect of preimmunization in each combination tested.

To further define the effect of immunization, mixed leukocyte cultures were prepared in which donor cells were combined with mitomycin-C or X-irradiated host strain spleen cells for 5 days. At this time cell suspensions were washed and tested for their ability to generate an LIA reaction *in vivo*. In initial experiments heterologous plasma or serum (10% human plasma; 10% fetal calf serum) was used, leading to nonspecific activation of cultured spleen cells. We therefore turned to fully homologous culture medium, containing 0.5% syngeneic mouse serum (cf. 3), and in this serum the nonspecific activation of cultured cells did not occur.

The results of *in vitro* immunization of donor spleen cells are shown in Table II, representing 1 of 3 experiments yielding similar results. A marked effect of *in vitro* stimulation was observed: 0.2×10^6 BALB/c spleen cells, cultured in the presence of Ha/ICR stimulator cells, are able to induce a strong response in Ha/ICR animals, while they are ineffective in the unrelated C57BL/6 strain at this low cell number. As can be seen from the data, moreover, cultured, unstimulated control cells are inactive at this concentration. The response of *in vitro* stimulated cells can be obtained using one tenth as many cells as are required when freshly isolated, unimmunized donor cells are assayed, suggesting that in vitro activation, proliferation and/or selection for specific effector cell populations has taken place.

TABLE I. EFFECT OF PRIOR IMMUNIZATION OF BALB/c MICE ON THE ABILITY OF THEIR SPLEEN CELLS TO EVOKE LIA REACTIONS IN ALLOGENEIC HOSTS[a]

Immunizing strain	Recipient strain	No. of animals	$\bar{X}$ No. of vessels ± SE
A.			
none	DBA	8	8 ± 0.6
DBA	DBA	8	30 ± 2.6
C57BL/6	DBA	8	16 ± 1.2
B.			
none	C57BL/6	8	17 ± 1.0
C57BL/6	C57BL/6	8	30 ± 1.3
C_3H	C57BL/6	8	22 ± 1.3
C.			
none	C_3H	7	14 ± 1.2
C_3H	C_3H	7	28 ± 2.0
DBA	C_3H	7	20 ± 2.2
D.			
none	Ha/ICR	10	28 ± 3.4
Ha/ICR	Ha/ICR	10	39 ± 2.8

[a] Background values (syngeneic cell inoculations) were always <3.0.

TABLE II. EFFECT OF *In Vitro* STIMULATION BY Ha/ICR CELLS ON THE CAPACITY OF BALB/c SPLEEN CELLS TO INITIATE LIA REACTIONS *In Vivo*.

	Host animals		
Donor cells	BALB/c	C57BL/6	Ha/ICR
Unstimulated[a]	1.3 ± 0.7[b]	4.4 ± 0.7	2.7 ± 0.6
Stimulated[a]	3.6 ± 0.7	7.4 ± 1.2	33.7 ± 4.8
Freshly Isolated[c]	0.4 ± 0.4	11.5 ± 1.2	14.3 ± 0.8

[a] 0.2×10^6 cells.
[b] $\bar{X}$ number of vessels ± SE; $n = 8$ for each value.
[c] 2.0×10^6 cells.

The time course of *in vitro* activation of effector cells was studied by examining the ability of cells removed from MLC cultures at various intervals after explantation to evoke LIA. The experiments (data not shown) indicate that while some effect of MLC activation was seen at 48 hr, maximum effectiveness was observed after 5 days.

Preimmunization of host animals. In order to examine whether immunization of host animals would interfere with subsequent LIA activity Ha/ICR mice were immunized against BALB/c spleen cells and then tested for their responsiveness to BALB/c, CBA and C57BL/6 donor allogeneic cells. The pooled results of several experiments are shown in Table III. They demonstrate that preimmunization leads to a specific reduction in responsiveness to allogeneic cells of the immunizing phenotype.

As a more rigorous test of this finding we next examined simultaneously the effect of immunization against C57BL/6, BALB/c or both C57BL/6 and BALB/c cells. The results

TABLE III. EFFECT OF PREIMMUNIZATION OF Ha/ICR MICE TO BALB/c CELLS ON THEIR SUBSEQUENT VASCULAR RESPONSE TO ALLOGENEIC EFFECTOR CELLS.

Donor strain	Response of Ha/ICR recipients[b]	
	Immunized	Unimmunized
BALB/c[a]	(33) 8 ± 0.8[c]	(36) 27 ± 1.2
CBA	(24) 22 ± 1.4	(20) 25 ± 1.2
C57BL/6	(10) 25 ± 0.9	(16) 25 ± 1.0

[a] 2×10^6 spleen cells were injected intradermally.
[b] Mice were irradiated with 750R X-rays 2–24 hr prior to injection of effector cells.
[c] Data given as mean # of vessels ± SE; number in parentheses indicates total number of tests made.

of a single experiment are shown in Table IV. The data show that preimmunization against donor cells leads to a specific decrease in the LIA reaction evoked by spleen cells from that strain, without interfering with the LIA reaction evoked by donor cells from an unrelated strain. In addition it can be seen that immunization against two distinct allogeneic strains leads to additive effects, i.e. mice immunized against both BALB/c and C57BL/6 cells show reduced responsiveness to both BALB/c and C57BL/6 cells presented singly or in combination, without any reduction in responsiveness to cells of the unrelated CBA strain.

Discussion. Angiogenesis can be induced by a variety of cells including cells obtained from solid tumors (cf. 4, 5), from regenerating antlers (6), from the corpus luteum (7), from epidermal cells (8) and from the adult male salivary gland (4). With respect to the reticuloendothelial system angiogenic activity has been demonstrated for specifically stimulated lymphocytes (1, 6, 9, 10), for syngeneic embryonic yolk sac cells (9) and for nonspecifically activated macrophages (11, 12). A variety of angiogenesis-inducing factors have also been described.

Our earlier work demonstrated that the LIA reaction involved a host response resulting from a local graft-versus-host reaction occurring when immunocompetent T-lymphocytes were injected intradermally into semi-allogeneic or allogeneic irradiated host animals. In now demonstrating that preimmunization of donor animals leads to a specific increase of the ability of spleen cells from such animals to elicit a vascular reaction it is noteworthy that in the strain combination not involving H-2 disparity (Table I, group A) the ratio of number of vessels induced by specifically sensitized cells/number of vessels induced by unsensitized cells was 4, while in all H-2 incompatible combinations (groups B, C, D) this ratio did not exceed two. These observations support the earlier findings of Simonsen with respect to a low "factor of immunization" where H-2 incompatibility was involved (13). The nonspecific (or cross-reactive) effect, measured as the ratio of number of vessels induced by immunization to a strain unrelated to the host/number of vessels induced by specific immunization, ranged from 33–42% (cf. Table I), a figure consistent with the level of cross reactivity reported in the literature (3, 14).

The finding that in vitro immunization was a more efficient means of increasing spleen cell effector activity than in vivo immunization is similar to observations made on the generation of cytotoxic T-cells (15). On the other hand, our observation (unpublished) that maximal LIA reactivity can be achieved when histocompatibility differences involve only the I-region indicate that the LIA reaction is not dependent on the generation of cytotoxic effector cells.

In interpreting the observations that preimmunization of the host results in a lowered LIA response it should be kept in mind that host animals were sensitized only one time and were also heavily irradiated. We cannot assume, however, that the host anti-effector cell activity does not involve a radiation-sen-

TABLE IV. EFFECT OF PREIMMUNIZATION ON THE ABILITY OF Ha/ICR MICE TO RESPOND TO ALLOGENEIC EFFECTOR CELLS: TESTS FOR SPECIFICITY AND FOR ADDITIVE EFFECTS IN THE LIA ASSAY SYSTEM.[a]

Donor strain[b]	Immunizing strain			
	C57BL/6	BALB/c	C57BL/6 + BALB/c	none
C57BL/6	14 ± 0.9[c]	23 ± 2.4	11 ± 1.2	24 ± 2.7
BALB/c	26 ± 1.4	9 ± 1.5	11 ± 0.9	25 ± 2.1
C57BL/6 + BALB/c	29 ± 2.0	19 ± 1.2	15 ± 2.1	44 ± 4.3
CBA	25 ± 1.5	22 ± 3.2	20 ± 2.1	22 ± 1.6

[a] Ha/ICR recipients were irradiated with 750r X-rays 2 hr prior to inoculation of test cell suspensions.
[b] 2×10^6 splenocytes; in combination studies (BALB/c + C57BL/6) 2×10^6 of each suspension were injected.
[c] Data presented as $\bar{X}$ number of vessels ± SE; 6 assays/group.

sitive cell, (e.g. cytotoxic T-cell) since we examined effector cell activity within a few hours of irradiation of host animals. It would be helpful if one could delay LIA assays for several days after irradiation, but this is precluded because of the critical role played by host radiation-sensitive stimulator cells (16, 17 and unpublished observations).

In general, our results of host immunizations are parallel to those reported for the Normal (Direct) Lymphocyte Transfer Reaction (cf. 16, 18) where preimmunization of host animals tended to reduce the skin thickening, erythema, and associated histological changes induced by the intradermal injection of allogeneic immunocompetent lymphocytes.

There is increasing evidence that the LIA reaction may depend on the production of soluble mediators (lymphokines), since both supernatants obtained from cultures of antigen-stimulated lymph node cells (19) and supernatants obtained from mixed leukocyte cultures (unpublished observations) can evoke angiogenesis. Whether this lymphokine activity is in turn due to secondary activation of macrophages (cf. 11, 12), involves a direct induction of endothelial cell migration and proliferation, or includes yet other causal mechanisms remains to be determined.

Summary. We have previously shown that immunocompetent lymphocytes can induce angiogenesis following intradermal inoculation into allogeneic or semi-allogeneic host animals. Experiments are described demonstrating that preimmunization of donor animals leads to a specific increase in angiogenesis-inducing capacity of effector spleen cells, while preimmunization of host animals results in a selective, specific reduction in lymphocyte-induced vascular changes.

The results are discussed in terms of the increasing evidence that lymphocyte-induced angiogenesis is mediated by soluble factors (lymphokines) released following lymphocyte activation.

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