LYMPHOCYTE-INDUCED ANGIOGENESIS: A QUANTITATIVE AND SENSITIVE ASSAY OF THE GRAFT-VS.-HOST REACTION*

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While progress in achieving quantitative measures of cellular immune responses has been rapid since the advent of various in vitro correlates of cell-mediated immunity (1-6), the need for a quick and precise measurement of such responses in vivo remains. Such measures as mean survival time after injection of immunocompetent cells into adult allogeneic irradiated hosts (cf. 7), for example, or mean graft survival time after skin transplantation suffer from excessive variability, length of time required for completion of the assay, and procedural difficulties. The induction of splenomegaly in neonatal recipients of allogeneic cells (8) is reasonably quantitative but operationally restrictive. Other assays such as enlargement of draining lymph nodes (9) or hepatic lymphoid infiltration (10), although more sensitive and more directly reflecting numbers of effector cells, are limited in applicability; the latter, moreover, requires histology. Local graft-vs.-host reactions have also been assessed by damage to the host kidney after injection of allogeneic immunocompetent cells under the kidney capsule (11), and by skin reactions elicited by intradermal inoculation of such cells (12); the latter has been called the normal lymphocyte transfer reaction (12, 13).

The normal lymphocyte transfer reaction seemed to us to have the greatest potential advantage for an in vivo assessment of immunological disparity between donor and host. The extensive studies of Brent and Medawar (12, 13) and of Billingham and his colleagues (14, 16) established the reaction as reflecting a local graft-vs.-host reaction. Their assessment of the strength of reaction was based on measurements of a local swelling, induration of the skin and erythema; other adjuncts of inflammation have also been reported (17, 18). Our own work has been directed at assessment of the host local vascular response which under appropriate experimental conditions can serve as a rapid, reliable, and quantitative measure of the activity of intracutaneously injected immunocompetent lymphocytes.

Materials and Methods

Animals. Mice were from our own colony-included BALB/cAu, C57BL/6JAu, C3H/HeAu, BALB/c × C3H (F1), and nu/nu backcrossed for 5+ generations to BALB/cAu. Additional animals obtained commercially included C3H and BALB/c (Charles River Laboratories, Wilmington, Mass.), C57BL/10 and C57BL/10D2 (Jackson Laboratories, Bar Harbor, Maine) and C57BL/6. DBA/2, BDF1

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and HalCR/SCH (ARS Sprague Dawley). In general, donors and recipients were adult males, 2-4 mo old; exceptions are noted in the text.

**Cell suspensions.** Donor cell suspensions were obtained in normal saline by teasing and abrasion against a stainless steel screen. Further disruption of clumps was achieved by passing the cell suspension in and out of a syringe. Cell suspensions were washed twice in saline, viability was assessed by trypan blue exclusion, and the cell concentration was then appropriately adjusted.

**Irradiation.** Irradiation was carried out with a GE Maxitron X-ray machine (General Electric Co., West Lynn, Mass.) operating at 300 kvp, 20 mA, dose rate of 83 R/min in air, with 1 mm Cu and ½ Al added filtration. Unless otherwise indicated, animals were irradiated with 800 R no more than 24 h before injection of test cell suspensions. Donor cell suspensions were irradiated in saline.

**Cell Transfer.** Intracutaneous injections were performed with a 27 gauge needle which was introduced about ½ cm into the skin as close to the epidermis as possible. 0.1 ml of volume was injected in all instances. Experiments designed to validate the methodology indicated that indeed up to eight injection sites per animal could be used (cf. Table I A), and that the various sites did not yield significantly different results; only the mid-dorsal and mid-ventral regions need to be avoided. In most experiments, however, animals received only four injections.

It was noticed that occasionally one recipient was a stronger or weaker responder than most of the animals used in a given experiment. For this reason inoculation of all experimental groups into each animal was favored, and this type of protocol was used throughout our experiments.

**Vascular Response Assay.** Mice were killed by ether, and a mid-ventral incision was made in the skin which was then separated carefully from the underlying muscles. Further steps were carried out with the aid of a dissecting microscope. Scoring of all of the reaction areas was carried out at the same magnification (× 1) and only vessels readily detected at this magnification were counted. Weaker responses, examined under higher magnification, would otherwise give erroneously high counts. Transmitted light was used to examine albino mice, but direct light had to be used on mice with dark skin. The sites of injection, recognized by local swelling or discoloration, were exposed by carefully removing fat or other tissue covering the area. Most recently we have found that addition of a drop of trypan blue to the cell inoculum so that the cellsuspension becomes lightly colored, simplifies subsequent location of the sites of injection. Gentle stretching of the skin with forceps also helps visualization of the reaction area.

All extra blood vessels connected with the scar region, and contrasting with the normal background vasculature, were counted; these vessels were readily detected because of their tendency to loop formation and tortuosity. The change in shape of vessels ranged from minimal zigzagging to formation of a complete loop depending on the degree of histoincompatibility and the time of assay, but each extra branch was counted as one regardless of its state of deformity. Additional looped vessels were occasionally seen farther away from the injection site, but these were not included in the count because they were considered too variable to be included as a meaningful measure of the response under study. The counting procedure may be more readily understood by referring to Fig. 1 a-c and Fig. 2.

Once the assay guidelines were established, technicians could read the reaction within ± 10%. However, virtually all data included in the present report were counted by the same person (Y. A. Sidky). To assure absence of bias representative experiments for each series were carried out using coded suspensions; where feasible coding of host animals was also used.

**Presentation of data.** No experiments is included in more than one table. In some instances a correction factor of 0.85 has been applied to day 3 data to permit pooling of day 2 and day 3 measurements. Calculation of this factor was based on the means of 50 consecutive experimental groups for which both day 2 and 3 data were obtained.

**Other.** Corticosteroid-resistant thymocytes were produced by intraperitoneal injection of 2.5 mg hydrocortisone acetate 2 days before utilization of the thymus. Mitomycin-C treatment was carried out by incubation of cells for 30 min with 25 ug/ml of mitomycin-C (Sigma Chemical Co., St. Louis, Mo.), followed by careful washing.

## Results

**General Description.** Transfer of spleen cells into the skin of adult irradiated and nonirradiated recipients results in the appearance of a thickened yellowish
FIG. 1. (a) $2 \times 10^8$ BALB/c spleen cells were injected into irradiated HaICR mice 3 days before assay. Since the reaction is three-dimensional, the photograph does not show all vessels with the same degree of clarity. ($\times 10$). (b) Drawing of the reaction site shown in Fig. 1 a. S indicates scar region. Blood vessels in region A and the upper part of region F are not considered part of the reaction. Counts: A = 0; B = 6; F = 4. For counts of C, D, and E see Fig. 1 c. (c) Detail of region surrounding scar (see also Fig. 2). Counts: C = 16–18; D = 4; E = 11.
scar at the site of injection, which is readily detected after 24 h irrespective of the genetic compatibility or incompatibility between donor and host animals. When isologous spleen cells are inoculated (2 x 10⁶-4 x 10⁶ cells were routinely injected as controls) a scar was visible at the site of injection; this scar persisted for 2-4 days. The scar was sometimes reddish, but few, if any extra blood vessels were

FIG. 2. 2 x 10⁶ allogeneic cells (BALB/c-HaICR), day 3. Same region as shown in Fig. 1 b.
FIG. 3. 4 x 10⁶ isogeneic cells (HaICR), day 2. x26.
FIG. 4. 8 x 10⁶ allogenic cells (BALB/c-HaICR), day 2. Halo around scar region. x11.
FIG. 5. As Fig. 4, 1 day later. Halo has cleared and extensive vascular response is now seen. x11.
directly associated with the scar and their number and appearance did not change over a 6-day observation period (Fig. 3).

When immunocompetent cells are injected into histoincompatible hosts, however, the scar region becomes surrounded by an intricate network of blood vessels which in spite of its complexity, can be characterized in quantitative terms (Figs. 1 and 2). By enumerating distinct vessels, i.e. by counting every discrete vascular branch arising by divarication, it is possible to assign a number value to the vascular reticulation induced by foreign lymphocytes. The results, presented in Tables I-X, indicate that the number thus determined bears a direct and meaningful relationship to the number of cells injected, immunological state of competence of the injected cells, and the degree of histoincompatibility between donor and recipient.

Effect of Cell Concentration. While cell concentration studies were carried out with numerous strain combinations, the most extensive series of experiments was performed using BALB/c donor spleen cells assayed in HaICR host animals. This line is genetically uniform as determined by nonstimulation in mixed leukocyte culture and by retention of skin grafts (19). The use of this strain combination for many of our experiments was dictated by the ready availability of animals, ease of scoring, and the virtually complete absence of background vasculature in animals injected with syngeneic cells.

### TABLE I A

| Animal no. | Cell dose \( \times 10^{-8} \) | 0.2 | 0.5 | 0.75 | 1.0 | 2.0 | 3.0 | 4.0 | Syn.† |
|------------|-------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Assay: day 2 |                               |     |     |     |     |     |     |     |     |
| I          |                               | 0   | 9   | 13  | 21  | 26  | 29  | 27  | 0   |
| II         |                               | 0   | 12  | 15  | 18  | 26  | 27  | 3   | 0   |
| III        |                               | *   | 13  | 18  | 20  | 25  | 29  | 31  | 2   |
| IV         |                               | *   | 11  | 15  | 19  | 21  | 31  | 30  | *   |
| V          |                               | *   | 7   | 16  | 18  | 23  | 28  | 30  | 0   |
| VI         |                               | *   | 8   | 13  | 19  | 24  | 25  | 28  | 0   |
| VII        |                               | 4   | 14  | 20  | 30  | 35  | 37  | 45  | 0   |
| Assay: day 3 |                               |     |     |     |     |     |     |     |     |
| VIII       |                               | *   | 27  | 30  | 37  | 45  | 47  | 36  | *   |
| IX         |                               | 3   | 17  | 21  | 32  | 39  | 44  | 43  | 3   |
| X          |                               | 0   | 17  | 19  | 25  | 31  | 33  | 35  | *   |
| XI         |                               | 4   | 14  | 20  | 22  | 25  | 29  | 32  | 0   |
| XII        |                               | 0   | 17  | 29  | 32  | 35  | 46  | 52  | 2   |
| X          |                               | 0.6 | 10.6| 15.7| 20.7| 25.7| 29.4| 32.2| 0.3 |

Single experiment; each animal received eight injections.

* Not found
†† \( 2 \times 10^6 \) HaICR spleen cells
§ Not injected
TABLE I B

Effect of Cell Dose on Number of Vessels Induced by BALB/c Spleen Cells Infected Intracutaneously into Irradiated HaICR Mice

| Cell dose (× 10^{-e}) | $\bar{X}$ vessels ± SE | Assay day 2* | Assay day 3§ |
|------------------------|------------------------|-------------|-------------|
| 1                      | 16.7 ± 1.0             | 16.8 ± 2.8  |             |
| 2                      | 25.5 ± 1.3             | 25.3 ± 3.3  |             |
| 4                      | 29.3 ± 2.3             | 23.3 ± 3.4  |             |
| 6                      | 33.3 ± 2.1§             | > 60        |             |
| 7                      | 31.2 ± 1.6§             | > 60        |             |
| 8                      | 29.3 ± 1.8§             | > 60        |             |
| 10                     | 34.2 ± 2.5§             | > 60        |             |
| Syngeneic              | 0.8 ± 0.5             |             |             |

Single experiment; each animal received eight injections.
* Six animals/group.
† Four animals/group.
§ Halo around scar region.

TABLE I C

Effect of Cell Dose on Number of Vessels Induced by BALB/c Spleen Cells Injected Intracutaneously into Irradiated HaICR Mice

| Cell dose (× 10^{-e}) | No. of animals | $\bar{X}$ vessels ± SE |
|------------------------|---------------|------------------------|
| 0.2                    | 19            | 1.47 ± 0.40            |
| 0.5                    | 26            | 9.42 ± 0.62            |
| 0.75                   | 29            | 13.24 ± 1.19           |
| 1.0                    | 28            | 22.43 ± 0.82           |
| 2.0                    | 12            | 26.08 ± 1.08           |
| 3.0                    | 6             | 31.50 ± 1.72           |
| 4.0                    | 12            | 37.50 ± 1.72           |
| Syn.*                  | 107           | 0.15 ± 0.01            |

Three additional experiments, consecutive, assay day 2.
* Syngeneic cells at doses from $2 \times 10^6$ to $4 \times 10^6$; all experiments included.

The results of a single experiment involving BALB/c spleen cells injected into HaICR mice are presented in Table I A. In this experiment each animal was given eight separate injections, and animals assayed on day 2 or day 3 are listed individually to illustrate the type of experimental design and results obtained. A second experiment, involving higher numbers of cells, is summarized in Table I B. Table I C includes the pooled results of three additional experiments. The results presented for the BALB/c-HaICR combination are consistent with results obtained for a variety of other strain combinations, only some of which are listed in Table II.
The tables show that the number of looped vessels increases linearly with increased number of inoculated immunocompetent cells up to $1 \times 10^6$ cells. Injection of more cells, up to $4 \times 10^6$, caused a progressively smaller increase of looped vessels. The use of higher concentrations of spleen cells ($6 \times 10^4$–$10 \times 10^4$ cells) was not reflected in an increase in the number of vessels observed on day 2 (Table I B). Rather the injection led to the formation of a bigger scar, thickening and induration of the skin, and the appearance of a red halo around the site of injection (Fig. 4, cf. also 14). The presence of a halo either prevented the formation or detection of more vessels than observed at lower concentrations of cells; once this halo had cleared on the following days, however, an extensive network of blood vessels was observed (Fig. 5, cf. Table I B).
Time-Course of Vascular Responsiveness. A variety of strain combinations were employed in assessing the progressive changes of the vascular pattern with time. The pattern was similar for all combinations studied (Table III). In the case of strong histocompatibility differences, the effect of injecting allogeneic cells could be detected within 24 h, but the vascular bed appeared faint and looping was minimal. During the second and third day, the number of deformed vessels increased and their pattern became clearly discernible. Subsequent to this time, the amount of looping was found to increase, but quantitation of the response became more difficult. In general, injection of $3 \times 10^6$ cells or less resulted in a maximum response by day 3 or 4, while above that concentration additional vessels and vascular contortions could be observed until day 6. A limited number of small hemorrhages (petechiae) tend to appear at those reaction sites which persist to the sixth or seventh day at which time almost all the reactions have already declined. From this it appears that readings made 2-3 days after inoculation are optimal for measuring the induced vascular responses for the cell doses most frequently used in the present study.

Effect of Irradiation of the Host on Vascular Responsiveness. Our original reason for irradiating host animals was to prevent a host-vs.-graft reaction in allogeneic strain combinations. However, it readily became apparent that irradiation served to reduce variability and increase the number of blood vessel divarications not only in allogeneic but in semiallogeneic combinations as well (Fig. 6).

Effect of Irradiation and Mitomycin-C Treatment of Donor Cell Suspensions on Their Ability to Induce Angiogenesis. When competent donor cells were irradiated in vitro with 1,000 R X-rays, a significant response was nonetheless apparent 48 h after inoculation (Table IV). However, this response was a transient one and the number of vascular branches decreased rapidly thereafter. Similarly, when donor lymphocytes were treated with mitomycin-C before transfer the response over the first 2 days was normal but again there was a rapid decline after this time.

Distribution of Cells Competent to Induce Angiogenesis. A survey was carried out to ascertain the efficacy of cells from various lymphoid organs to
induce a vascular response. Spleen, lymph node, thymus and bone marrow cells from individual animals were compared, as shown in Table V. Lymph node and spleen cells both induced a strong response, while thymus and bone marrow were unable to do so. Hydrocortisone-resistant thymocytes, on the other hand, showed competence equivalent to that of spleen cells.

Since these experiments suggested that the effector cell population was of thymic origin or thymus-dependent, spleen cells from thymusless (nu/nu) mice were studied and compared to spleen cells from their littermates. The experiments showed that spleen cells from thymusless animals are not capable of evoking a host vascular response (Table VI).

The ontogeny of spleen cell competence to induce angiogenesis was also examined. Newborn spleen cells lacked adequate numbers of effector cells. 4 days after birth, however, sufficient numbers of competent spleen cells were present to mount a weak but detectable response, as shown in Table VII.

**Genetic Aspects of Lymphocyte-Mediated Angiogenesis.** A variety of strain
TABLE V
Organ Distribution of BALB/c Cells Competent to Induce Angiogenesis in HaICR Host Mice (Representative Experiment)

| Source of cells* | Assayed after: | 2 days§ | 3 days¶ |
|-----------------|----------------|---------|---------|
|                 |                | 0 ± 0   | 0 ± 0   |
| Bone marrow     |                | 1.5 ± 0.7 | 0.5 ± 0.5 |
| Thymus          |                | 19.8 ± 1.0 | 19.8 ± 1.8 |
| Spleen          |                | 28.5 ± 1.3 | 39.2 ± 3.1 |
| Lymph node      |                | 16.0 ± 1.4 | 9.0 ± 0.5 |
| Steroid thymus‡ |                | 1.0 ± 0.5 | 0 ± 0   |
| HaICR spleen    |                |         |         |

* 2 x 10⁶ cells.
‡ Hydrocortisone-resistant thymocytes. Although 2 x 10⁶ cells were used, in this experiment the thymus was larger than usual, i.e., steroid treatment was not fully effective. In other experiments 0.4 x 10⁶ hydrocortisone-resistant thymus cells were as effective as 3 x 10⁶ spleen cells.
§ Six animals/group.
¶ Four animals/group.

TABLE VI
Ability of Spleen Cells from Congenitally Athymic (nu/nu) BALB/c Mice to Induce Angiogenesis in Various Strains of Mice: Pooled Experiments

| Host strain | C3H | HaICR | BDF₁ |
|-------------|-----|-------|------|
| Source of donor cells | X vessels ± SE | X vessels ± SE | X vessels ± SE |
| nu/nu       | 1.9 ± 0.9 (6)* | 0.3 ± 0.3 (12) | 0.0 (8) |
| nu/+ or +/+  | 17.9 ± 3.1 (5) | 20.6 ± 3.0 (11) | 10.5 ± 2.1 (8) |
| BALB/c      | 20.5 ± 2.9 (4) | 21.7 ± 2.7 (11) | 18.1 ± 2.7 (8) |

* No. of mice in parentheses.

combinations were used throughout the studies already reported, all conforming to the pattern of a graft-vs.-host reaction. Thus, positive reactions were obtained both in allogeneic combinations and in semiallogeneic ones where donor parental cells were injected into recipient F₁ cells, on the other hand, did not generally cause a reaction in parental strains. Reciprocal experiments designed to test this point specifically are shown in Table VIII.

To assess the role of H-2 disparity, combinations between C57BL/10 and C57BL/10D2 were carried out. The experiments show that congenic resistant lines differing only in the major histocompatibility region (in this case B vs. D) cause a vigorous vascular response (Table IX).

In combinations involving BALB/c and DBA/2 (H-2 compatible, M disparate) a low but detectable response was obtained (Table X). It should be pointed out, moreover, that in addition to seeing fewer vascular branches, the degree of vessel
**TABLE VII**

**Ontogeny of Ability of BALB/c Spleen Cells to Induce Vascular Response in HaICR Recipients: Representative Experiment**

| Age of donor* | No. of animals | $\bar{X}$ vessels ± SE |
|---------------|----------------|------------------------|
| Newborn       | 10             | 0 ± 0                  |
| 4-day old     | 15             | 14.4 ± 1.3             |
| 8-day old     | 10             | 13.8 ± 1.7             |
| Adult         | 10             | 23.0 ± 2.2             |
| Syngeneic adult | 10           | 0.8 ± 0.6              |

* $2 \times 10^8$ cells.

**TABLE VIII**

**Graft-vs.-host Nature of Induced Angiogenesis: Reciprocal Experiments between Parental and F₁ Mice (Representative Experiment)**

| Source of donor cells* | Host strain     | $\bar{X}$ vessels ± SE |
|------------------------|-----------------|------------------------|
|                        | C57BL/6         |                        |
| C57BL/6                | 1.5 ± 0.7 (8)†  | 24.7 ± 2.3 (6)         |
| BDF₁                   | 3.1 ± 0.9 (8)   | 0.9 ± 0.6 (6)          |
| BALB/c                 | 21.5 ± 1.8 (15) | 24.3 ± 1.1 (12)        |

* $2 \times 10^8$ cells. Correction factor applied for day 3 observations.
† No. of mice in parentheses.

**TABLE IX**

**Lymphocyte-mediated Angiogenesis Induced by Spleen Cells Differing only at the H-2 Region: Pooled Experiments**

| Source of donor cells* | Host strain     | $\bar{X}$ vessels ± SE |
|------------------------|-----------------|------------------------|
|                        | C57BL/10        |                        |
| C57BL/10               | 1.4 ± 0.4 (14)† | 21.3 ± 2.1 (13)        |
| C57B10:D2              | 17.8 ± 1.8 (13) | 1.0 ± 0.4 (13)         |

* $3 \times 10^8$ cells. Correction factor applied for day 3 observations.
† No. of mice in parentheses.

deformity is also less when a non- $H$-2 difference is involved, and the decline of the reaction is rapid.

**Discussion**

Our experiments describe a new and sensitive assay for the effect of intracutaneous administration of immunocompetent lymphocytes into the skin of immunized hosts (the normal lymphocyte transfer reaction). The assay is based on the
measurement of induced vascular reticulation. We have named this response lymphocyte-induced angiogenesis (LIA) for reasons to be discussed below. The assay is rapid, with accurate assessment of the reaction possible within 48 h after injection of immunocompetent cells; it is capable of detecting in vivo the action of as few as $5 \times 10^5$ spleen cells differing at the $H$-2 locus; it can detect some non-$H$-2 differences such as M locus disparity; and it shows dose-dependent, quantifiable effects.

As is the case for the previously described normal lymphocyte transfer reaction, LIA is a manifestation of the graft-vs.-host reaction. This conclusion is supported by the following findings: (a) allogeneic or semiallogeneic cells readily induce angiogenesis while syngeneic cells fail to do so; (b) $F_1$ cells injected into parental-strain animals are unable to induce significant angiogenesis; (c) at concentrations where spleen and lymph node cells can induce a response, thymus and bone marrow cells are ineffective; (d) on the other hand the small number of hydrocortisone-resistant cells found in the thymus show strong reactivity; (e) cells from thymusless (nu/nu) donors cannot induce a vascular response, while their littermates initiate a normal response; (f) combinations between BALB/c and DBA/2 result in a weak but detectable response; (g) the newborn spleen does not contain LIA-competent cells but competent cells appear by day 2 and gradually increase in relative frequency.

It should be kept in mind that the LIA assay, as is the case for pock-formation on the chorioallantoic membrane (20) or splenomegaly induced in neonatal animals (8) relies on amplification by the host in response to graft-vs.-host activity of immunocompetent lymphocytes. A host response also represented a major element in previous measures of the normal lymphocyte transfer reaction, such as swelling, erythema and induration of the skin (12-16), or increased vascular permeability as measured by Evans blue leakage (18); since the LIA assay measures only the vascular pattern of the host, however, it appears to offer a greater opportunity for analysis of the reaction.

In this connection, the observation that irradiation of recipient animals before lymphocyte transfer increases the reliability and sensitivity of the LIA assay should be discussed. Ramseier and Billingham (15) found that irradiation of

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**Table X**

Vascular Response to Immunocompetent Cells in the Absence of H-2 Incompatibility: BALB/c vs. DBA/2

| Source of donor cells* | Host strain |     |     |
|------------------------|-------------|-----|-----|
|                        | BALB/c      | DBA/2 |
| BALB/c                 | 0.5 ± 0.4 (7)$ | 11.0 ± 0.5 (6) |
| DBA/2                  | 8.7 ± 1.9 (6) | 0.0 (6) |
| C57BL/6                | 26.7 ± 1.9 (6) | 39.3 ± 4.1 (6) |

*3 x 10^8 cells.
†No. of mice in parentheses.

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$1$ Abbreviation used in this paper: LIA, lymphocyte-induced angiogenesis.
hamsters before lymphocyte transfer impaired the reaction, and a similar observation was made with respect to the host kidney response to a local graft-vs.-host reaction (11). Since the LIA assay measures an endothelial response, however, the known radio-resistance of resting endothelial cells combined with the increased vascular permeability induced by irradiation (21) would appear to be most favorable. At the same time host irradiation, by destroying host immunocompetent cells, permits the use of allogeneic donors without the complicating factors of a host-vs.-graft reaction.

It is most encouraging that relatively low numbers of cells are needed to elicit detectable vascular reticulation. Indeed, when higher numbers of effective cells are injected locally the vascular response rapidly becomes obscured by induration and erythema; it is this aspect of the response that was investigated so extensively by Streilein and Billingham (14) who injected $10^7$ cells in their study of the normal lymphocyte transfer reaction. Since LIA can be detected after inoculation of as few as $5 \times 10^5$ cells, a highly active mediator of endothelial response is indicated.

While our experiments show that $H-2$ differences are sufficient to evoke LIA, we have not yet further dissected the $H-2$ region to ascertain the relationship of ability to evoke vascular reticulation to the Ir, LD, or other subsections of that region; such experiments are in progress. The fact that differences between BALB/c and DBA/2 are read as weak reactivities is encouraging in the light of related findings reported by Salaman et al. (22) and by Auerbach and Shalaby (23), and suggests that the host response to transfer of normal lymphocytes is not entirely restricted to $H-2$ incompatible combinations as suggested by Streilein and Billingham (14).

Our finding that mitomycin-C treatment or irradiation of donor lymphocytes does not abrogate the initiation of a vascular response, but does prevent its continuation can be interpreted to reflect on the need for cell division for amplification, and/or to the rapid removal of treated cells from the site of injection, presumably by host phagocytes. If the later response to normal lymphocytes is based on a different effector cell population it may of course be possible that such a population of cells is indeed more radiosensitive than the one initiating LIA. Brent and Medawar (12, 13) suggested that the normal lymphocyte transfer reaction proceeds in two phases, a recognition phase within the first two days which does not require cell division, and flareup phase which occurs subsequently and which is eliminated in the absence of cell division.

In vitro studies have shown that lymphocytes, in response to the first phase, produce a variety of mediators or soluble factors (cf. 4, 24), such as migration inhibition factor, mitogenic factor, blastogenic factors, macrophage-activating factor, skin-reactive factor, and interferon (6, 24); such mediators may well provide the appropriate stimulus for host endothelial cell responses, thus acting as an angiogenesis factor as well and leading to the observed alterations in vascular pattern.

The described skin reaction can probably be applied to other types of delayed hypersensitivity, e.g., the tuberculin reaction, other bacterial antigens, serum proteins or other simple protein antigens, etc. (25). Preliminary experiments on mice sensitized with sheep red blood corpuscles strongly suggest that indeed the
resulting delayed hypersensitivity (26) can be measured by our assay protocol; sensitized mice should not be irradiated however. We have also been able to use the hamster as a recipient, and it has been possible to assay for activity of xenogeneic cells.

The similarity between the vascular response induced by foreign lymphocytes and that induced by grafted tumor tissue or tumors in situ is striking and intriguing. Operationally, tumor-induced angiogenesis (cf. 27, 29) and lymphocyte-mediated vascular reticulation are indistinguishable. Moreover, in experiments to be reported elsewhere (footnote 2) we have observed that lymph node grafts placed in assay situations developed for tumor angiogenesis studies, i.e. intracorneal grafts in rabbits and chorioallantoic membrane grafts in chick embryos, are capable of evoking host angiogenesis and the kinetics of these responses appear comparable. Furthermore in these assays there is clear evidence for angiogenesis as distinguished from changes in vascular visualization through constriction and vasodilation, which could certainly play a significant role in the observed intradermal responses. It is of interest in this connection that irradiation of tumor cells also fails to inhibit their ability to initiate angiogenesis in vivo (30) or to release tumor angiogenesis factor in vitro (31). The meaning of the similarity between these two systems remains enigmatic, although the role of a common mediator of angiogenesis is certainly a distinct possibility.

The possible interrelationship between lymphocyte-induced angiogenesis and tumor-induced angiogenesis bears further consideration. Aside from the possible direct action of a tumor-originating mediator of angiogenesis (TAF) (27), could there be an additional vascular response resulting from lymphocyte recognition of tumor antigens? Such a reaction could act synergistically, and its absence may well restrict the rate of tumor progression. Indeed the contradictory findings in studies of tumor growth in nu/nu mice (cf. 32) and the restriction of tumor growth and metastasis after a graft-vs.-host-induced lymphopenia (33) may find their explanation in just such lymphocyte-mediated amplification of angiogenesis.

Aside from the extensive vascular changes associated with delayed hypersensitivity and inflammation, a number of disease states exist in which neovascularization plays an integral part (e.g. diabetic retinopathy). The demonstration that thymus-derived lymphocytes can cause an increase in local vascularization as reflected both in the number and tortuosity of vessels associated with the activity of those cells may well prove to be significant in this respect. Similarly, the observed increased size of placental mass and vasculature in histoincompatible fetal-maternal combinations (34) and especially after previous immunization of the mother against paternal antigens suggests that LIA may be a meaningful response in a variety of physiological conditions requiring vascular adaptation.

A number of experiments are in progress to further define the nature of LIA: to establish the pattern of ontogeny of immunocompetence as measured by this reaction; to ascertain the potential of using the vascular response as an indirect measure of immune reactivity between mixtures of xenogeneic cell populations (bystander assay); to define more precisely the source of target cells responding to the lymphocyte-derived stimulating material; and to characterize the nature

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1 Kubai, L., Y. Sidky, J. Folkman, and R. Auerbach. Unpublished observations.
LYMPHOCYTE-INDUCED ANGIOGENESIS

of material released by lymphocytes undergoing a local graft-vs.-host reaction. We expect that these experiments will increase our understanding of the mechanism and significance of vascular changes mediated by immunocompetent lymphocytes.

Summary

A new and sensitive assay for the effect of intracutaneous administration of immunocompetent lymphocytes into the skin of irradiated unimmunized mice is described. The assay, which we have termed lymphocyte-induced angiogenesis (LIA) involves enumeration of new vascular branches induced by the action of these competent cells. As is the case for the previously described normal lymphocyte transfer reaction, LIA is a manifestation of the graft-vs.-host reaction, as shown by experiments utilizing appropriate genetic combinations. The reaction is dose-dependent, and within the dose range of $2 \times 10^5 - 4 \times 10^6$ cells the number of vessels induced correlates with the number of immunocompetent cells injected. At these dose levels spleen, lymph node, and hydrocortisone-resistant thymocytes are effective; bone marrow and thymus cells are not. Spleen cells from nude mice are incapable of inducing LIA, while mitomycin-C and irradiated lymphocytes can initiate but not maintain the reaction. The relationship between lymphocyte-induced angiogenesis and tumor-induced angiogenesis has been discussed as have the implications of these findings to delayed hypersensitivity, inflammation, and vascular pathology.

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