MIGRATORY BEHAVIOR OF LYMPHOCYTES WITH SPECIFIC REACTIVITY TO ALLOANTIGENS

II. Selective Recruitment to Lymphoid Cell Allografts and Their Draining Lymph Nodes*

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The development of a normal immune response depends, in part, on the interaction of antigen with one or more lymphocyte subpopulations with specific reactivity to that antigen. Presumably as a means of assembling and concentrating the proper admixture of these lymphocytes with antigen, rodents are able to selectively recruit circulating, specifically reactive lymphocyte to sites of sequestered antigen. It has been firmly established that specifically reactive lymphocytes are selectively recruited to the spleen after an intravenous (i.v.) challenge (1-4). More recently, I have demonstrated a selective recruitment of immunospecific lymphocytes to the bone marrow and liver as well as the spleen in mice challenged i.v. with semiallogeneic spleen cells (5) or xenogeneic red blood cells (Emeson, unpublished data). In mice challenged subcutaneously in the four paws with chicken red blood cells (CRBC) or sheep red blood cells (SRBC), anti-CRBC or anti-SRBC lymphocytes are selectively recruited to the lymph nodes draining the challenged paws (6). The migratory patterns of circulating immunospecific lymphocytes at the sites of cell-mediated immune lesions and their draining lymph nodes are still somewhat controversial (7-10).

We have recently developed a dual-antigen, dual-isotope assay in our laboratory that enables us to monitor the migratory behavior of specific antiallogeneic lymphocytes with a high degree of precision (5). In the following studies, I have used this assay to delineate the migratory pattern of specific antiallogeneic lymphocytes of mice given subcutaneous injections of semiallogeneic spleen cells into their paws. The data obtained indicated that after a subcutaneous challenge into the paw, specifically reactive lymphocytes were selectively recruited to their paws and local draining lymph nodes and deleted from their circulating blood and nondraining nodes.

Materials and Methods

Animals. Genetically inbred female DBA/2J (DBA), (C3H/HeJ × DBA/2J)F1 (CDF) and

* Supported by grant CA 1746 awarded by the National Cancer Institute, Department of Health, Education, and Welfare.

1 Abbreviations used in this paper: cpm, counts per minute; CRBC, chicken red blood cells; dpm, disintegrations per minute; HBSS, Hanks' balanced salt solution; i.v., intravenously; L, left; NMS, normal mouse serum; PBL, peripheral blood leukocytes; R, right; SRBC, sheep erythrocytes; SRL, specifically recruited lymphocytes; TCA, trichloroacetic acid.
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GENERATION OF 3H & 14C-LABELED ANTIALLOGENEIC LYMPHOCYTES

ADOPTIVE TRANSFER

SYNGENEIC DBA RECIPIENTS CHALLENGED IN FOOT PADS

Fig. 1. Diagrammatic outline of the experimental design (see text for details).

EXPERIMENT 1. Dose response. The basic experimental design for experiments 1, 2, and 4 is diagrammatically outlined in Fig. 1. 3H-Labeled anti-C3H/HeJ (C3H) and 14C-labeled anti-C57BL/6J (C57BL) lymphocytes of DBA origin were generated in the spleens of lethally irradiated (900 rad) CDF and BDF mice, respectively, as previously described in detail (5), pooled, and adoptively transferred to six groups of four DBA recipients. The mice of each of the first four groups had been challenged 6 h previously by a subcutaneous injection of four, threefold serial dilutions of CDF spleen cells into the two right paws and similar injections of BDF spleen cells into the two left paws. All four paws of the fifth and sixth groups of mice were injected with Corynebacterium parvum and Hanks' balanced salt solution (HBSS), respectively, to serve as additional controls. After 48 h each recipient was sacrificed, its right and left paws and their draining lymph nodes segregated into right and left samples, and each sample analyzed radiochemically.

EXPERIMENT 2. Reversal of the radioactive labels. This experimental design was similar to that of experiment 1 except that the radioactive labels of the adoptively transferred cells were reversed (anti-C3H lymphocytes were labeled with 14C and anti-C57BL lymphocytes labeled with 3H) and only one dose of spleen cells (2.0 × 10⁷ cells) was used to challenge the recipient's paws.

EXPERIMENT 3. Time sequence of response. This experimental design was similar to that of experiment 1 except that the 3H-labeled anti-C3H and 14C-labeled anti-C57BL were adoptively transferred to two groups of 16 DBA recipients. The mice of one group had been challenged 6 h previously by a subcutaneous injection of 2.0 × 10⁷ CDF spleen cells into each of their four paws, and the mice of the other group by a similar injection of BDF spleen cells into each of their four paws. After 24, 48, 72, and 96 h, subgroups of four mice from each group were sacrificed, and their paws, local draining lymph nodes, nondraining nodes (mesenteric), spleen, liver, bone marrow, lungs, small intestines, and peripheral blood leukocytes (PBL) analyzed radiochemically.

EXPERIMENT 4. Characterization of surface membrane markers of the selectively recruited cells. The experimental design was similar to that of experiment 1 except that the pools of 3H-labeled anti-C3H and 14C-labeled anti-C57BL lymphoid cells were enriched for the presence of T lymphocytes by the use of a nylon column technique or B lymphocytes by the use of anti-Thy 1.2 ascitic serum plus complement.

Irradiation. Mice were irradiated in an acrylic plastic box by a Cobalt 60 teletherapy unit (courtesy of Krystyne Okoniewski and Dr. C. Botstein, Department of Radiotherapy, Montefiore Hospital and Medical Center). The 900 rad dose was given at a rate of 71.9 R/min at a distance of 80 cm.

Challenge. In experiment 1, mice were challenged subcutaneously in the foot pads with 0.21 × 10⁶-6.0 × 10⁷ CDF or BDF spleen cells per paw. In experiments 2, 3, and 4, 2.0 × 10⁷ spleen cells were adopted as the standard challenging dose. Semiallogeneic cells were used as the challenging antigen to avoid the added effects of a graft vs. host reaction. Selected control groups
received 0.350 mg of *C. parvum* (generously provided by Dr. John K. Whisnant, Burroughs Wellcome Co., Research Triangle Park, N.C.) or HBSS, respectively, in each paw. All injections were given in a 50-μl vol with a tuberculin syringe and 27 gauge needle.

**Preparation of Cell Suspensions for Challenge.** Spleens were minced in HBSS, passed through a series of graded stainless steel screens, and washed three times as previously described (11). Red blood cells were lysed with 0.83% NH₄Cl buffered at 7.2 with Tris (12).

**Preparation of Cell Suspensions for the Adoptive Transfer.** Spleens of CDF and BDF mice were pooled separately and processed as above with the following changes: (a) after the first wash, the cells were freed of most of the dead cells by the method of von Boehmer and Shortman (13); (b) red blood cells were not removed from these suspensions; and (c) in experiment 4, the cells of each pool were enriched for the presence of T or B lymphocytes as previously described (5). In all experiments, the two pools of spleen cells were combined and given i.v. in equal portions (about 2.0 × 10⁶ viable cells per mouse) to the previously challenged recipients. The donor to recipient ratio in all experiments was approximately 1:1.

**Preparation of Tissues for Scintillation Counting.** (a) Paws. The four paws of each mouse were amputated at approximately 5 mm above the ankle joint and digested in N NaOH (1 ml/paw) at 37°C for 1-1.5 h (i.e. until most of the soft tissues of the paws were visibly digested). In experiments 1, 2, and 4, the two right paws and two left paws of each mouse were segregated and processed separately. In experiment 3, all four paws of each mouse were processed together. After the alkaline digestions, the resulting mixtures were then centrifuged at 400 g for 5 min, and the digests separated from the remaining bone fragments. The bone fragments were washed with an additional 2-4 ml N NaOH, again centrifuged, and the wash combined with the above digest. The digest was then brought to a concentration of 10% trichloroacetic acid (TCA) by the addition of 40% TCA and allowed to stand for 1 h at 4°C. The resulting precipitate was washed in successive changes of absolute ethanol with 0.2% sodium acetate, absolute ethanol, and ether, dried at 50°C and its DNA extracted relatively free of protein and RNA by the method of Orlov and Orlova (14). (b) Draining lymph nodes. The axillary, brachial, and popliteal nodes of each mouse were carefully freed of fat, soaked in three daily changes of acetone at room temperature, and digested in N NaOH without extracting their DNA. In experiments 1, 2, and 4, the lymph nodes of the right and left sides of each mouse were segregated and processed separately. In experiment 3, both the right and left axillary, brachial, and popliteal nodes of each mouse were processed together. (c) Nondraining lymph nodes. The mesenteric lymph nodes were removed completely and processed as in b. The mesenteric nodes represent approximately 65% of the total nondraining lymph node complement. The spleens, bone marrows, lungs, small intestines, and PBL were processed as previously described (5).

**Counting of Samples.** The above samples were digested in 1.0 ml of N NaOH and suspended in a dioxane Cab-O-Sil (Packard Instrument Co., Inc., Downers Grove, Ill.) cocktail as previously described (11) and the counts per minute (cpm) ³H and ¹⁴C obtained simultaneously in a Beckman LS-350 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.). The efficiencies of the ³H and ¹⁴C and the crossover of ¹⁴C counts into the ³H channel were determined by the addition of ³H- or ¹⁴C-toluene internal standards (New England Nuclear, Boston, Mass.).

**Treatment of Data and Statistical Analyses.** The cpm ³H and ¹⁴C were converted to disintegrations per minute (dpm, 15), and the ratios dpm ³H/dpm ¹⁴C were determined for each sample. The proportion of anti-C3H to anti-C57BL lymphocytes contained in a tissue or portion of tissue from each group or subgroup was inferred from its mean ³H/¹⁴C ratio. In experiments 1 and 4, the relative differences in the ³H/¹⁴C ratios of the paws and draining nodes of the right and left sides of each animal were compared by dividing the ³H/¹⁴C of the right side (R) by the ³H/¹⁴C of the left side (L). A one-tailed t test for dependent samples was then used to determine whether the mean R/L ratios of the tissues of each group differed significantly from 1.00. Mean R/L ratios significantly greater than 1.00 indicated the relative degrees of selective recruitment present, whereas mean R/L ratios significantly less than 1.00 indicated the relative degrees of a selective depletion or "negative recruitment" present. In experiment 2, because the radioactive labels were reversed, the ³H/¹⁴C ratios of the tissues were compared by dividing the ³H/¹⁴C of the left side by the ³H/¹⁴C of the right side. Differences in the mean ³H/¹⁴C ratios of the tissues between paired subgroups of mice challenged with different alloantigens (experiment 3) were evaluated statistically by a two-tailed t test for independent samples. To estimate the relative differences
in the mean $^{3}\text{H} / ^{14}\text{C}$ ratios of the tissues of those paired subgroups, the mean $^{3}\text{H} / ^{14}\text{C}$ ratios of the tissues of each subgroup of CDF-challenged mice were divided by the mean $^{3}\text{H} / ^{14}\text{C}$ ratios of the tissues of its paired subgroup of BDF-challenged mice (CDF/BDF). CDF/BDF ratios above 1.00 indicated selective recruitment, whereas those below 1.00 indicated negative recruitment. The use of one-tailed $t$ tests was justified in experiments 1, 2, and 4 because preliminary experiments enabled me to make more critical null hypotheses for the paws and their draining nodes for these experiments.

An estimate of the absolute value of specifically recruited lymphocytes (SRL) in each tissue of mice challenged with C3H alloantigens was made as previously described (5) by use of the formula $\text{SRL}_{\text{C3H}} = (^{3}\text{H}) - (^{14}\text{C} \times \text{R control})$. For each tissue $\text{SRL}_{\text{C3H}}$ equals specifically recruited anti-C3H lymphocytes, $^{3}\text{H}$ and $^{14}\text{C}$ equals the dpm $^{3}\text{H}$ and $^{14}\text{C}$, respectively, and (R control) equals the mean $^{3}\text{H} / ^{14}\text{C}$ ratio of the theoretical control group. The factor $(^{14}\text{C} \times \text{R control})$ provides an estimate of the amount of $^{3}\text{H}$ retained in the tissue by virtue of nonspecifically recruited lymphocytes.

Results

Dose Response. The mean R/L value of the paws and draining nodes of the groups of mice challenged with all four doses were similar and all significantly (P < 0.01) greater than 1.00 (Table I), indicating that the recruitment of specific antiallogeneic lymphocytes to the paws and their draining nodes was at least in part immunologically specific for all four doses. The mean R/L values in the paws and draining lymph nodes of mice challenged with only HBSS or $C. \text{parvum}$ did not differ significantly (P > 0.10) from 1.00. The top two doses had similar capacities to recruit SRL to the paws, which were significantly greater than those of the two lower doses. At the same time, the top three doses were approximately equally capable of recruiting SRL to the draining nodes. When doses of $2.0 \times 10^7$ and $2.0 \times 10^4$ semiallogeneic cells were used in subsequent experiments to challenge recipients, there were slight but significant (P < 0.01) degrees of selective recruitment (R/L = 1.14 ± 0.044 and 1.05 ± 0.010, respectively) to the draining nodes, but not (R/L = 1.02 ± 0.023 and 0.97 ± 0.024, respectively) to the paws (Emeson, unpublished data). The dose $2.0 \times 10^7$ cell was adopted as the standard challenging dose for subsequent experiments.

Selective Recruitment of Antiallogeneic Lymphocytes with the Radioactive Labels Reversed. To provide evidence that the demonstration of selective recruitment of antiallogeneic cells to the paws and draining nodes was not on account of an unusual antigen or isotope effect, I repeated the previous experiment with $2.0 \times 10^7$ semiallogeneic spleen cells as the challenging doses, but with the radioactive labels of the donor pool reversed (i.e. anti-C57BL lymphocytes labeled with $^{3}\text{H}$ and anti-C3H lymphocytes labeled with $^{14}\text{C}$). The mean L/R ratios of the paws and their draining nodes, significantly above 1.00 in each of two experiments (Table II), indicated, as in the previous experiment, the presence of selective recruitment of specific antiallogeneic lymphocytes to both of these tissues.

Selective Recruitment and Deletion of Antiallogeneic Lymphocytes 24, 48, 72, and 96 h after Challenge. In this experiment, all four paws of each of two separate groups of mice were challenged with either CDF or BDF spleen cells. This experimental design provided a means of determining the effects of selective recruitment to the paws and their draining nodes on the migratory
TABLE I
Recruitment of ³H-Labeled Anti-C3H and ¹⁴C-Labeled Anti-C57BL Lymphocytes to the Paws and Draining Nodes of DBA Mice 48 h after a Subcutaneous Challenge with Varying Doses of Semiallogeneic Spleen Cells

| Cell dose × 10⁷ | Paws | Draining lymph nodes |
|----------------|------|---------------------|
|                | Mean R/L ± SE* | Mean SRL ± SE† | Mean R/L ± SE* | Mean SRL ± SE† | Total SRL‡ |
| 6.0            | 1.23 ± 0.031   | 870 ± 159         | 1.96 ± 0.053   | 840 ± 149       | 1,710      |
| 2.0            | 1.19 ± 0.015   | 530 ± 57          | 2.01 ± 0.127   | 860 ± 196       | 1,390      |
| 0.63           | 1.16 ± 0.005   | 220 ± 43          | 1.81 ± 0.071   | 594 ± 119       | 814        |
| 0.21           | 1.17 ± 0.018   | 230 ± 26          | 1.63 ± 0.067   | 240 ± 21        | 470        |
| C. parvum only | 1.00 ± 0.018   | 5 ± 65            | 1.00 ± 0.032   | -10 ± 62        | -5         |
| HBSS only      | 1.05 ± 0.038   | 7 ± 6.3           | 1.02 ± 0.051   | 4 ± 9.5         | 11         |

* Mean ³H/¹⁴C ratio of tissues of the right side divided by the mean ³H/¹⁴C ratio of the tissues of the left side. Four mice in each group.
† An estimate of the quantity of lymphocytes recruited by virtue of their immunological specificity in the CDF-challenged (right side) paws and draining nodes. SRLCDF = (³H) - (¹⁴C × R control).
‡ SRL paws plus SRL draining lymph nodes.

TABLE II
Recruitment of ¹⁴C-Labeled Anti-C3H and ³H-Labeled Anti-C57BL Lymphocytes to the Paws and Draining Nodes of DBA Mice 48 h after a Subcutaneous Challenge with Semiallogeneic Spleen Cells

| Experiment | Paws | Draining lymph nodes |
|------------|------|---------------------|
|            | Mean L/R ± SE* | Mean SRL ± SE† | Mean L/R ± SE* | Mean SRL ± SE† | Total SRL |
| 1          | 1.15 ± 0.009 | 150 ± 6            | 1.86 ± 0.088 | 270 ± 33       | 420       |
| 2          | 1.13 ± 0.015 | 300 ± 63           | 1.68 ± 0.042 | 250 ± 24       | 550       |

* Mean ³H/¹⁴C ratios of tissues of the left side divided by the mean ³H/¹⁴C ratio of the tissues of the right side. Four mice in each group.
† An estimate of the quantity of lymphocytes retained by virtue of their immunological specificity in the BDF-challenged (left side) paws and draining nodes. SRLBDF = (³H) - (¹⁴C × R control).

behavior of specific antiallogeneic lymphocytes to other tissues. The mean ³H/¹⁴C ratios of the paws and draining nodes of CDF-challenged mice were significantly greater at almost all of the time intervals than those of BDF-challenged mice (Table III), again indicating the presence of selective recruitment to both of these tissues. The relative differences between the mean ³H/¹⁴C ratios (CDF/BDF) of the paws of the two subgroups seen at 48, 72, and 96 h after challenge were similar, whereas those of the draining nodes were greatest at 48 h after challenge. The accumulation of SRL in the paws peaked at 48 h after challenge and declined slightly over the next 48 h. The accumulation of SRL in the draining nodes peaked sharply at 48 h and then fell off rapidly over the ensuing 48 h.

Significantly lower mean ³H/¹⁴C ratios were noted in the nondraining lymph nodes (mesenteric) at 24 (P < 0.10), 48 (P < 0.01), and 96 h (P < 0.05) and blood
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Recruitment of \(^{3}H\)-Labeled Anti-C3H and \(^{14}C\)-Labeled Anti-C57BL Lymphocytes to the Paws and Draining Nodes of DBA Mice 24, 48, 72, and 96 h after a Subcutaneous Challenge with Semiallogeneic Spleen Cells

| Time (h) | Paws Mean \(^{3}H/^{14}C\) ± SE in mice challenged by: | SRL ± SE | Draining lymph nodes Mean \(^{3}H/^{14}C\) ± SE in mice challenged by: | SRL ± SE |
|---------|---------------------------------|----------|---------------------------------|----------|
|         | CDF/BDF*                        | CDF      | BDF                             | CDF/BDF* |
|         | CDF BDF                         |          |                                 |          |
| 24      | 9.51 ± 0.112 9.24 ± 0.103 1.03   | 230 ± 170| 9.91 ± 0.038 7.91 ± 0.259 1.26   | 490 ± 73 |
| 48      | 9.14 ± 0.022 8.19 ± 0.066 1.12   | 530 ± 24 | 9.48 ± 0.106 5.65 ± 0.105 1.68   | 1,120 ± 64|
| 72      | 8.59 ± 0.056 7.64 ± 0.113 1.12   | 490 ± 51 | 7.63 ± 0.127 5.16 ± 0.204 1.52   | 360 ± 26 |
| 96      | 8.17 ± 0.054 7.23 ± 0.183 1.13   | 450 ± 23 | 6.41 ± 0.083 4.87 ± 0.167 1.32   | 160 ± 20 |

* Mean \(^{3}H/^{14}C\) ratios of the tissues of CDF-challenged mice divided by the mean \(^{3}H/^{14}C\) ratios of the tissues of the BDF-challenged mice.

† An estimate of the quantity of lymphocytes recruited by virtue of their immunological specificity in the paws and draining lymph nodes of the CDF-challenged mice. SRL\(_{CDF} = \left(\frac{^{3}H}{^{14}C}\right)_{CDF} - \left(\frac{^{3}H}{^{14}C}\right)_{BDF} - \left(\frac{^{3}H}{^{14}C}\right)_{R control}.

‡ Probability differences in CDF- and BDF-challenged mice are due to chance <0.01 using a two-tailed t test for independent samples.

of all four time intervals of CDF-challenged mice as compared with those of BDF-challenged mice. These differences indicated a selective deletion or "negative recruitment" of antiallogeneic lymphocytes retained in these tissues at these time intervals. These differences were greatest at 48 h after challenge and have been confirmed at this time interval in two additional experiments. The differences in mean \(^{3}H/^{14}C\) ratios of the spleens, bone marrows, livers, small intestines, and lungs were generally small and inconsistent at all four time intervals.

**Total dpm \(^{3}H\) in the Tissues of Mice Challenged with CDF Cells.** To provide an estimate of the total radioactive label (and thus presumably an estimate of the total number of labeled lymphoid cells) retained in the various tissues of the CDF-challenged mice, the total dpm \(^{3}H\) (× 10\(^{-2}\)) of these tissues are given in Table IV. At 24 h the largest quantity of \(^{3}H\) was retained in the spleen (22,800 dpm), the next largest quantities in the challenged paws (16,500 dpm) and small intestines (11,400 dpm), and from 4,300-4,600 dpm in the draining nodes, marrow, liver, and lungs. The mean dpm \(^{3}H\) values seen at 24 h generally declined rapidly over the next 72 h in the paws, nondraining nodes, spleen, liver, lungs, and blood, and tended to remain stable in the draining nodes, marrow, and small intestines.

**Characterization of the Surface Membrane Markers of the Selectively Recruited Cells by Using Labeled Donor Cells Enriched for the Presence of T or B Lymphocytes.** When labeled donor cells were treated with normal mouse serum (NMS) plus complement (control cells), the mean R/L values and mean SRL of the paws and draining nodes of mice challenged 48 h previously (Table V) were similar to those in previous experiments (Table I). R/L values of the draining nodes, but not the paws, of mice receiving nylon column separated labeled donor lymphocytes were significantly increased as compared with those of mice receiving control cells. Mean SRL values of both the paws and draining nodes of these mice appeared to increase, considering the fact that the portions
**TABLE IV**

*Total dpm $^3$H Retained in Tissues of DBA Mice 24, 48, 72, and 96 h after a Subcutaneous Challenge with CDF Spleen Cells*

| Mean dpm $^3$H ± SE ($\times 10^{-2}$) | Paw | Draining nodes | Blood | Spleen | Bone marrow | Liver | Small intestine | Lungs |
|---------------------------------------|-----|----------------|-------|--------|-------------|-------|----------------|-------|
| h                                     |     |                |       |        |             |       |                |       |
| 24                                    | 165 ± 20.6 | 46.2 ± 5.30 | 25    | 228 ± 5.4 | 61 ± 4.8 | 43.5 ± 2.55 | 114 ± 25.5 | 45.4 ± 2.54 |
| 48                                    | 119 ± 7.9  | 49.1 ± 1.30 | 13    | 98 ± 9.0  | 61 ± 2.5  | 19.5 ± 1.61 | 91 ± 6.7  | 17.8 ± 1.06 |
| 72                                    | 71 ± 3.4   | 19.0 ± 1.06 | 7.5   | 81 ± 4.2  | 59 ± 3.2  | 14.9 ± 0.46 | 108 ± 4.5 | 14.8 ± 4.05 |
| 96                                    | 79 ± 5.6   | 11.8 ± 0.49 | 4.8   | 60 ± 2.7  | 40 ± 3.3  | 18.6 ± 1.68 | 64 ± 9.6  | 9.5 ± 1.5 |

* Each portion of donor pool cells given to each recipient contained 350,000 dpm $^3$H.

**TABLE V**

*Recruitment of Nylon Column Separated and Anti-Thy 1.2 Treated $^3$H-Labeled Anti-C3H and $^1$C-Labeled C57BL Lymphocytes to the Paws and Draining Lymph Nodes of DBA Mice 48 h after a Subcutaneous Challenge with Semiallogeneic Spleen Cells*

| Treatment of labeled donor cells | Paws | Draining lymph nodes |
|----------------------------------|------|----------------------|
|                                  | Mean R/L ± SE | Mean SRL ± SE | Mean R/L ± SE | Mean SRL ± SE | Total SRL |
| Nylon column*                   | 1.23 ± 0.031 | 200 ± 47 | 2.49 ± 0.080 | 770 ± 49 | 970 |
| Anti-Thy 1.2†                   | 1.17 ± 0.029 | 65 ± 18 | 1.95 ± 0.081 | 290 ± 53 | 335 |
| NMS (Control)*†                 | 1.20 ± 0.013 | 260 ± 49 | 1.92 ± 0.101 | 660 ± 110 | 920 |

* Each portion of this donor pool given to each recipient of this group contained 160,000 dpm $^3$H. Four mice in each group.

† Each portion of each of these donor pools given to each recipient of these groups contained 315,000 dpm $^3$H.

of nylon wool-treated donor cells which these mice received contained about one-half the total dpm $^3$H as did the portions of control donor cells. When the labeled donor cells were treated with anti-Thy 1.2 ascitic serum plus complement, the R/L values of the paws and draining nodes did not differ significantly from those of mice receiving control cells. Mean SRL values of these animals, however, were decreased to 25% of control values in the paws and to 43% of control values in the draining lymph nodes, suggesting that at least 75% of the SRL retained in the paws and 57% of the SRL retained in the draining nodes were anti-Thy 1.2 susceptible and thus T lymphocytes. Although this data suggests that B lymphocytes are also selectively recruited to the challenged paws and their draining nodes, I cannot be certain that some of the selectively recruited cells that resisted inactivation by anti-Thy 1.2 were not null cells or T lymphocytes with little or no Thy 1.2 antigen on their surfaces. Because macrophages were almost completely eliminated from the nylon column separated lymphocytes, it would appear that the induction of selective recruitment does not require their presence in the donor pool. These experiments did not provide evidence for or against the participation of the recipients' macrophages in the process of selective recruitment.
Discussion

The present studies clearly establish that the immunospecific antiallogeneic lymphocytes are selectively recruited to lymphoid cell allografts and their draining lymph nodes. This phenomenon has been demonstrated by the use of our dual-antigen, dual-isotope assay in each of the 164 mice examined to date over the course of 12 separate experiments. Evidence that the observed differences in $^{3}$H/$^{14}$C ratio were due to selective recruitment and not to other mechanisms such as thymidine reutilization, sequestration of dead cells or cells with antigen, antibody or antigen-antibody complexes adsorbed on their surface membranes, has been presented in previous papers (5, 11, 16, 17). Because a specific clone of antiallogeneic lymphocytes actually constitutes a very small fraction of the total recirculating pool of lymphocytes, one of the essential requirements of our dual-antigen, dual-isotope assay is to limit the uptake of radioactive label to this specific clone. The inability to do so 100% effectively, as well as other previously enumerated sources of background noise (5), undoubtedly resulted in an underestimation of the degrees of selective recruitment actually present in these experiments.

Selective Recruitment of Allografts. A large variety of experiments, dating back to the early 1960's, have been performed to determine whether there is a preferential retention of specifically reactive lymphoid cells in lesions of cell-mediated immunity (reviewed in 7-10). In the best controlled of the earlier experiments, the investigators were unable to demonstrate a preferential retention of these cells (18). More recently, Tilney and Ford (19) using a dual-antigen, dual-isotope assay similar to ours, were able to demonstrate a small surplus of radioactively labeled specific lymphocytes in rat skin allografts, but not in their draining lymph nodes. In their studies they generated the specific antiallogeneic lymphoid cells by means of a host vs. graft reaction rather than a graft vs. host reaction. Lance and Cooper (20) demonstrated a three to sixfold increase in the localization of $^{125}$I-UDR-labeled $H2$-activated splenic or lymph node cells in specific skin allografts as compared with third-party allografts or isografts. This degree of specific localization was far in excess of that found in the present experiments or those of Tilney and Ford (19). Although it is true that Lance and Cooper were correct in their conclusions, it is also true, as pointed out by Tilney and Ford (19), Ford (9), and Allwood (10), that Lance and Cooper's results were subject to other interpretations and thus their studies would have benefited by the use of additional controls, such as the use of labeled cells sensitized to a third-party allograft in the donor pools. Sprent and Miller (17) have also recently shown that $^{51}$Cr- or $^{125}$I-UDR-labeled $H2$-activated T lymphocytes display a very slight tendency to selectively localize to specific skin, tumor cell, or lymphoid cell allografts. Their studies, in my opinion, were also subject to alternative interpretations and would also have benefited by the use of additional controls, similar to those suggested for the studies of Lance and Cooper. In regard to both of these latter studies, Allwood (10) has provided an excellent review of some of the problems of designing appropriate experiments for demonstrating antigen-specific recruitment. He concluded that the use of two antigens with a criss-cross design is necessary to provide adequate control of the nonspecifically recruited lymphocytes.
Selective Recruitment to Lymph Nodes Draining Allografts. Emeson and Thursh (11) have previously used a somewhat different dual-antigen, dual-isotope assay to demonstrate a small but statistically significant degree of selective recruitment of specific antiallogeneic lymphocytes in nodes draining skin allografts. These studies differed from the present ones in that the radioactive labels were restricted to the long-lived lymphoid cells of the donor pools, and the skin donors and recipients were H-2 compatible. Hay et al. (21) and Cahill et al. (22) have provided evidence in sheep that when allogeneic lymphocytes are infused directly into a lymph node via its cannulated afferent lymphatic vessel, the lymphocytes with specific reactivity to the alloantigens are selectively recruited to that lymph node.

Negative Recruitment to Blood, Nondraining Lymph Nodes, and Spleen. The negative recruitment of specific antiallogeneic lymphocytes in the blood and peripheral lymph nodes is in agreement with observations of other investigators who have demonstrated a selective deletion of these cells in the recirculating pool of lymphocytes of sheep when allogeneic lymphocytes are infused into a cannulated afferent duct of a lymph node (21) and in the blood (3), thoracic duct lymph (1, 2), and lymph nodes (23) of rodents challenged i.v. or intraperitoneally.

General Comments. Thus far in this report I have dealt almost exclusively with the subpopulations of lymphocytes that are recruited to challenged tissues by virtue of their immunological specificity. Emeson and Thursh (11) have previously suggested, but not proved, that immunologically specific recruitment depends, in part, on the fact that specifically reactive lymphocytes possess antigen-specific surface membrane receptors capable of binding to specific antigens within the challenged tissues. Lymphocytes that lack specific immunological reactivity to the challenging antigen are also found in large numbers at the sites of challenge and their draining nodes. This nonspecific type of recruitment (or trapping) has been extensively investigated by Dressor et al. (24), Zatz and Lance (25), Frost and Lance (26), Frost (27), Allwood (10), and more recently by Zatz (28, 29) and may help provide the "critical mass" of lymphocytes required for the cellular interactions known to be necessary to generate most immune responses. Both immunologically specific and nonspecific recruitment are presumably important in the immune response.

At the present time, there is no rigorous evidence that specifically reactive lymphocytes are actually attracted to, or home to, the site of sequestered antigen (9). It is more likely that they are selected from the normally heterogeneous pool of circulating lymphocytes and detained by sequestered antigen as they percolate through the tissue, whereas lymphocytes lacking specific reactivity to the antigen emigrate out of the challenged tissue (or allograft), at their normal rate. This process of selection is aided and abetted by the enormous increase in traffic of circulating lymphocytes through sites of antigenic challenge (30-34). Cahill et al. (34) have recently presented evidence obtained from sheep that the increased quantities of 51Cr-labeled lymphocytes (thus, predominantly nonspecific lymphocytes) found in antigen-challenged tissues and their draining lymph nodes is principally due to an increase in the traffic of recirculating lymphocytes passing through the tissues and nodes rather than to an increased transit time through the node. They conclude that
the term "lymphocyte trapping" is inappropriate, at least in regard to the lymphocytes that are recruited to the tissue nonspecifically. These authors correctly point out, however, that their experimental methods were not precise enough to exclude the possibility that the small proportion of lymphocytes with specific reactivity to the challenging antigen was actually retained or entrapped within the challenged node.

In the present studies the specifically recruited lymphocytes have been defined in terms of their possession of T- or B-surface membrane markers. They have not as yet been defined in terms of the functional heterogeneity known to exist within T- and B-cell populations. The use of antisera directed against Ly components of the T-cell surface, to selectively eliminate functional subsets of T lymphocytes from the labeled donor pool might provide further information in this regard (35-37).

The present studies, coupled with our previous ones (4-6), may be relevant to current attempts to evaluate the tumor-specific cell-mediated immune responses of cancer patients by assaying lymphocytes isolated from their circulating blood (reviewed in 38, 39). My studies indicate that for at least the first four days after an i.v. (5) or subcutaneous challenge with alloantigen, specific antiallogeneic lymphocytes are deleted from blood. If immune reactions to alloantigens have any relevance to immune reactions to tumor-specific antigens, my studies suggest that lymphocytes with specific reactivity to tumors would be found in greatest abundance in and around the tumors and their draining lymph nodes (analogous to a subcutaneous challenge with alloantigens). As tumor cells or tumor antigens are shed into the blood stream (analogous to an i.v. challenge with alloantigens), the relevant tumor-specific lymphocytes might also be selectively recruited to the spleen, liver, and marrow (5). In both instances, the blood may not be a good source of lymphocytes to assay for tumor-specific cell-mediated immunity. Sampling the blood may be analogous to playing a game of chance, with success representing the probability of sampling those few specifically reactive antitumor lymphocytes that happen to be in transit between lymphoid organs and tumor. Future studies in tumor immunology must not ignore the profound effects that antigen has on the migratory behavior of specifically reactive lymphocytes.

Summary

A dual-antigen, dual-isotope assay has been used to monitor the migratory behavior of selectively labeled antiallogeneic lymphocytes in mice challenged subcutaneously in all four foot pads with semiallogeneic spleen cells. \(^{3}H\)-labeled anti-C3H and \(^{14}C\)-labeled anti-C57BL lymphocytes of DBA/2J origin were pooled and adoptively transferred to multiple groups of previously challenged DBA/2J recipients. In some of the studies, separate groups of recipients were challenged with either CDF or BDF spleen cells in all four paws, whereas in others CDF spleen cells were used to challenge the right paws of each mouse in the group and BDF spleen cells to challenge the left paws of each mouse in the group. At intervals varying from 24 to 96 h after challenge, a subgroup of four mice from each appropriate group was sacrificed and the relative numbers of anti-C3H and anti-C57BL lymphocytes present in the challenged paws, draining lymph nodes, and other tissues of each mouse were inferred from the
mean $^3$H/$^{14}$C ratios of the respective tissues of that subgroup. The results of these studies firmly establish that specific antiallogeneic lymphocytes are selectively recruited to the paws and draining lymph nodes of mice challenged subcutaneously in the foot pads with semiallogeneic spleen cells and are deleted from their circulating blood and nondraining lymph nodes. A mechanism for antigen-induced selective recruitment and its possible functional significance in tumor immunology are discussed.

The expert technical assistance of Fred Weintraub is gratefully acknowledged.

Received for publication 8 July 1977.

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