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I. The Life-Span and Circulation Dynamics of the Immunologically Committed Lymphocyte*

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Acquired resistance to infection with intracellular bacterial parasites has its origin in an immunological mechanism in which specifically committed lymphoid cells have a crucial role (1). Cells with the capacity to protect normal mice (2) and normal rats (3) against infection with *Listeria monocytogenes* appear in the spleen and thoracic duct early in the course of a primary *Listeria* infection. The failure of the protective cells to adhere to glass or cotton, their presence in central lymph, and suppression by anti-lymphocyte globulin (4) imply that antimicrobial resistance is transmitted by a species of lymphocyte.

Although nearly all the cells in rat thoracic duct lymph qualify as lymphocytes, they vary with respect to size and structure, turnover, and possibly with respect to origin and immunological capacity (5, 6). The present radioautographic study was designed to measure the labeling characteristics and circulation dynamics of the cells which afford protection against a systemic *Listeria* infection. The results indicate that cells with this commission belong to a lymphocyte population that has a rapid turnover and a short-circulating life-span.

Materials and Methods

Animals.—The experimental subjects were male and female Lewis rats and members of the F_1 hybrid cross between these and rats of the DA strain. Donors of thoracic duct cells weighed 160–250 g; the recipients, in whom antimicrobial resistance was assayed, weighted 60–110 g.

Microorganisms.—*Listeria monocytogenes*, strain EGD, has been maintained by continuous passage in mice for the past 2 yr. Suspensions for immunization or challenge were prepared from 16 hr trypticase soy broth cultures of infected spleen.

Immunization.—Prospective donors of thoracic duct cells were specifically immunized against *L. monocytogenes* with living organisms injected subcutaneously into both hind footpads and over the lower abdomen. The total injection volume was 0.5 ml; it contained approximately 5×10^6 viable units.

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Measurement of Protective Immunity.—Protection against a challenge infection was measured in terms of the numbers of viable bacteria present in the liver and spleen at intervals after an intravenous injection of approximately 10^7 viable *L. monocytogenes.* The initial implantation of the challenge organism was obtained by plating homogenates of livers and spleens from four rats, sacrificed 1 hr after injection. Its subsequent behavior was determined in groups of four to six rats sacrificed 24, 48, and sometimes 72 hr after challenge.

Preparation of Lymphocytes.—Thoracic duct lymph was collected on the 7th day of a primary immunizing infection. The lymph from freshly cannulated donors was collected at 4°C into sterile flasks containing 5 ml of Ringer's solution, 100 units of heparin, and 60 μg of penicillin. Collections of 18–24 hr duration were obtained from several animals. These were pooled, filtered through surgical gauze to remove fibrin clots, and centrifuged for 10 min at 500 *g*. After washing once in tissue culture medium 199 (7) containing 1% fetal calf serum, the cells were resuspended in fresh medium at a suitable concentration for labeling or injection.

In experiments involving the injection of radioactively labeled cells, individual recipients were housed in a restraining cage (8) and given a continuous femoral vein infusion of Ringer's solution containing the appropriate nonradioactive nucleoside, delivered at the rate of 1.2 mg per day. The infusion began several hours before the injection of labeled cells and was continued to the end of the experiment.

Radioactive Labeling of Lymphocytes.—Thoracic duct cells were labeled in their DNA by injecting the intact animal with tritiated thymidine (^{3}H-thymidine, 3 Ci per mmole, New England Nuclear Corp., Boston, Mass.). In one experiment, thoracic duct cells were labeled also in their RNA by incubating the cells in vitro with tritiated uridine (^{3}H-5-uridine, 20 Ci per mmole, New England Nuclear Corp. The cells were suspended at a concentration of 5×10^7 cells per milliliter in medium 199 containing 5% fetal calf serum and 1 unit of heparin per milliliter. ^{3}H-5-uridine was added to give a final concentration of 2 μCi/ml and the mixture was incubated for 1 hr at 37°C in a water bath oscillating at 120 cpm. The cells were then washed once in nonradioactive medium and resuspended in fresh medium for injection. More than 95% of the cells prepared in this manner were viable, as judged by their appearance in the counting chamber and their ability to exclude trypan blue.

Enzyme Treatment.—Air-dried smears of lymphocytes were fixed for 10 min at 4°C in 90% v/v ethyl alcohol and 10% v/v glacial acetic acid. The cells were then treated for 1 hr at 37°C with the appropriate nucleases to remove RNA, DNA, or both. RNA was removed by incubating the cells in 0.025 M sodium phosphate buffer, pH 7.5, containing 0.5 mg/ml of RNAse (ribonuclease A, 3100 units/mg, Worthington Biochemical Corp., Freehold, N. J.); DNA was extracted in 0.025 M sodium barbiturate buffer, pH 7.5, containing 0.003 M magnesium sulphate and 0.1 mg/ml of DNAse (deoxyribonuclease I, 2500 units/mg, Worthington Biochemical Corp.). After treatment, the smears were rinsed for at least 30 min in running tap water.

Radioautography.—Fixed smears of lymphocytes were extracted for 20 min at 4°C with two changes of 5% trichloroacetic acid, then washed for at least 1 hr in cold running water. They were subsequently coated with a 2:1 dilution of K-5 nuclear research emulsion (Ilford Ltd., Ilford, Essex, England) and were exposed for either 2 or 6 wk. The radioautographs were stained after development with May-Grünwald–Giemsa stain.

RESULTS

Protective Capacity of Thoracic Duct Cells.—Thoracic duct cells obtained from rats on the 7th day of either a local or systemic infection with *L. monocytogenes* can protect normal recipients against a *Listeria* challenge (3). In the experiment recorded in Fig. 1, two groups of cell recipients and a group of

normal controls were challenged intravenously with 4.3×10^6 viable *Listeria* 1 hr before the transfer of thoracic duct cells from a panel of *Listeria*-infected donors. The cells were given intravenously in doses of 2×10^8 or 5×10^7 per

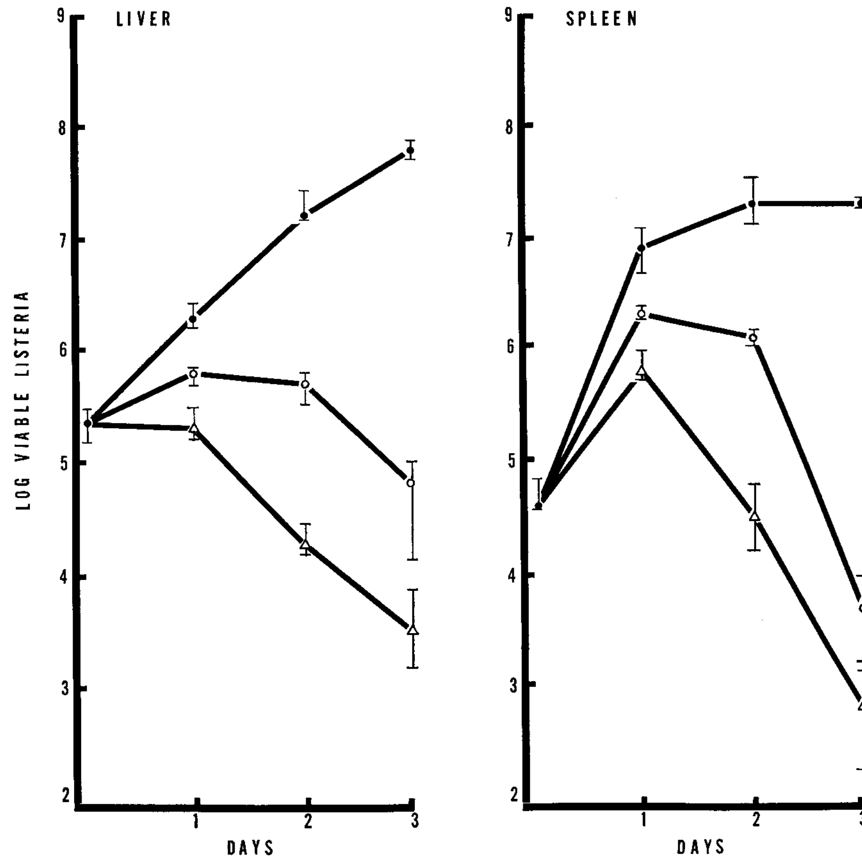

FIG. 1. Curves describing the growth of *L. monocytogenes* in the livers and spleens of normal rats (●——●) and rats protected with either 5×10^5 (○——○) or 2×10^6 (△——△) thoracic duct cells per gram of body weight. The cells were obtained from syngeneic donors on the 7th day of a primary *Listeria* infection. Means and ranges of five animals per time point.

100 g body weight. Their protective effect was expressed immediately in both liver and spleen and was greater with the larger dose of cells.

Production of Immunologically Committed Lymphocytes.—Cells which can protect normal rats against *L. monocytogenes* appear in the thoracic duct within 1 wk of a primary *Listeria* infection. Since they are not normally present there, they must be part of a cell population that is added to the lymph of infected

animals. The delivery of newly formed lymphocytes to the thoracic duct was therefore studied by injecting ^{3}H-thymidine (0.34 μCi per gram of body weight) into normal and acutely infected animals every 8 hr for 6 days. The numbers of cells recovered from the two groups on the 7th day were comparable, but varied greatly from animal to animal, mainly because the lymph flow from cannulated rats tends to be irregular. For this reason the output of labeled cells from normal and infected animals was not compared in absolute numbers. It is evident from Fig. 2, however, that the lymph of infected donors contained an increased percentage of radioactively labeled large and medium lymphocytes and a greatly increased percentage of labeled small lymphocytes. It is pre-

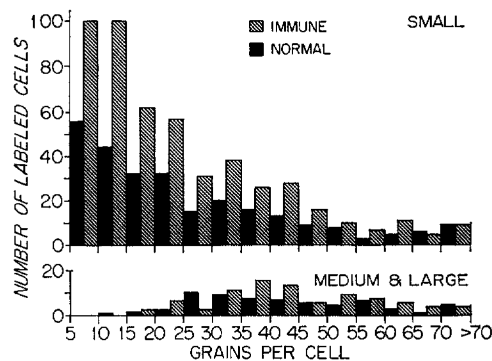

FIG. 2. Number of radioactively labeled lymphocytes in samples of 2000 cells distributed according to their grain counts. The subjects, three normal and three *Listeria*-infected rats, were injected repeatedly with ^{3}H-thymidine (see text). The lymph from infected animals contained an increased percentage of labeled lymphocytes but the distribution of radioactivity in the two populations was similar. Radioautographs exposed 6 wk.

sumably among this group that we should look for the cells which convey antimicrobial immunity.

Comparison of the Protection Afforded by Cells from Thoracic Duct Lymph of Actively and Adoptively Immunized Donors.—If the lymph-borne cells which can protect recipients belong to the pool of lymphocytes which recirculate from blood to lymph (9), it should be possible to recover them from the thoracic duct of an intermediate host. Cells from the thoracic duct lymph of *Listeria*-infected donors were therefore injected intravenously into a panel of normal intermediate hosts. Thoracic duct cells obtained from the inoculated animals 24–48 hr after injection were tested for their ability to protect a second group of normal recipients against a *Listeria* challenge. Table I indicates that the growth of *L. monocytogenes* was significantly inhibited in the livers and spleens of recipients given only 2×10^7 thoracic duct cells from a panel of actively immunized donors. Yet 10 times as many cells obtained from the thoracic ducts

of uninfected animals which had been adoptively immunized with 5×10^8 cells from the same donor source failed to influence a *Listeria* infection in a second panel of normal rats.

The failure of cells with protective properties to reappear in the lymph of adoptively immunized animals suggested that lymphocytes with a rapid turnover do not recirculate. This notion was tested in an experiment which measured the circulation dynamics and immunological performance of newly formed lymphocytes, including those generated during an immunizing infection. The method exploited the fact that thoracic duct cells can be labeled radioactively

TABLE I

Protective Capacity of Thoracic Duct Cells from Actively Immunized Donors and Adoptively Immunized Intermediate Hosts

Donor immunization	No. cells injected (10^6/100 g body wt)	Log_{10} protection*	
		Liver	Spleen
Active‡	50	1.20	0.81
	20	0.94	0.72
Adoptive§	200	−0.30	−0.01
	100	−0.58	−0.25
	50	−0.44	−0.17

* Difference in viable *Listeria* in tissue of five normal rats and groups of five rats given thoracic duct cells. In each case approximately 48 hr elapsed between the collection of cells from infected donors and i.v. challenge of the recipients with 10^7 *L. monocytogenes*. Animals sacrificed 48 hr after challenge. Using Duncan's multiple range test, all means in recipients of cells from actively immunized donors were found to be significantly different from those given cells from adoptively immunized donors at the $P < 0.01$ level.

‡ Infected with approximately 5×10^6 *L. monocytogenes*. Thoracic duct cannulated on 7th day of immunizing infection.

§ Injected i.v. with 5×10^8 thoracic duct cells from actively immunized donors. Cells collected from thoracic duct 24–48 hr after injection.

in both their DNA and RNA. Lymphocytes generated during the first 6 days of a primary *Listeria* infection were labeled in their DNA by injecting the animals repeatedly with ^{3}H-thymidine; those formed during (and before) immunization were labeled in their RNA by incubating the in vitro with ^{3}H-5-uridine (Fig. 3).

The injection schedule employed in this experiment (Table II) resulted in the radioactive labeling of nearly all the large and medium lymphocytes and 16.4% of the small lymphocytes; after incubation with ^{3}H-5-uridine, 84.6% of the small lymphocytes were labeled. The specificity of the label for DNA and RNA was confirmed by treating the cells with DNAse and RNAse, respectively.

Cells double-labeled in this manner were washed and injected intravenously into normal syngeneic recipients. In order to minimize reutilization of radioac-

tive material, each recipient was given a continuous intravenous infusion of Ringer's solution containing nonradioactive thymidine and nonradioactive uridine which was delivered at the rate of 1.2 mg per day (10, 11). Radioautographs prepared from the recipients' thoracic duct cells, collected over the 24

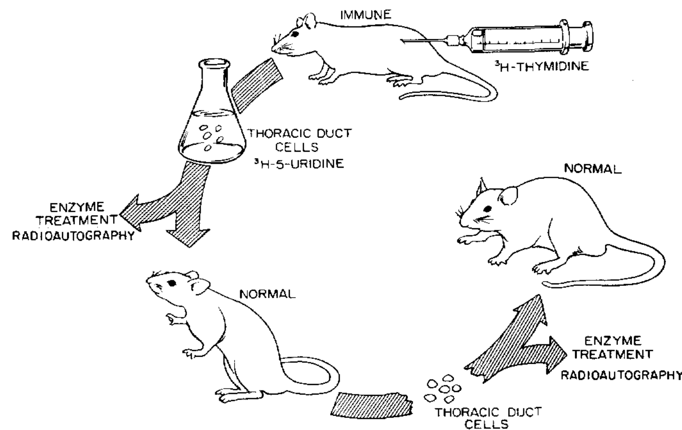

FIG. 3. Schematic diagram of experiment in which thoracic duct cells formed during and before a primary *Listeria* infection were compared with respect to their circulation dynamics and ability to transmit antimicrobial resistance through an intermediate host (see text).

TABLE II

Circulation Dynamics of Thoracic Duct Cells from Listeria-Infected Donors

Thoracic duct cells	Per cent lymphocytes labeled				Labeled small lymphocytes (RNA/DNA)
	Large and medium*		Small*		
	DNA	RNA	DNA	RNA	
Donors‡	98.5	>99.5	16.4	84.6	5.15
Recipients§	<0.5	0.5	1.1	20.3	18.45

* Radioautographs exposed 2 wk.

‡ Infected with approximately 8×10^6 *L. monocytogenes*. Animals injected every 8 hr for 6 days with 0.34 μCi of ^{3}H-thymidine per gram of body weight. First injection given at time of immunizing infection; lymph collected during first 24 hr after last injection. Pooled cells from three animals labeled in vitro with ^{3}H-5-uridine.

§ Injected i.v. with 7×10^8 thoracic duct cells from infected donors. Cells collected 24–48 hr after injection.

hr interval from 24 to 48 hr after injection, revealed that small lymphocytes were the only labeled cells which had recycled from the blood in substantial numbers. Evidently, the majority of these circulating donor cells had been formed before the immunizing infection, for most were labeled only in their RNA (Table II).

The few newly formed (DNA-labeled) small lymphocytes which did recirculate from the blood to the lymph were unable to influence a *Listeria* infection in a second panel of normal recipients. Table III shows that 5×10^8 thoracic duct cells from animals which had been adoptively immunized with

TABLE III

Protective Capacity of Thoracic Duct Cells from Actively Immunized Donors and Adoptively Immunized Intermediate Hosts

Donor immunization*	No. cells injected (10^6/100 g body wt)			Log_{10} protection‡	
	Total	DNA-labeled§	RNA-labeled§	Liver	Spleen
Active	200	47	169	1.48	1.02
	50	12	43	0.98	0.61
Adoptive	500	5	97	−0.14	−0.29

* See footnotes to Table II regarding immunization and cell labeling.

‡ Difference in viable *Listeria* in the livers and spleens of five normal rats and groups of five rats given thoracic duct cells. Cells injected intravenously 1 hr after i.v. challenge with approximately 10^7 *Listeria monocytogenes*. Animals sacrificed 48 hr after challenge. Using Duncan's multiple range test, all means were found to be significantly different from each other at the $P < 0.01$ level.

§ Radioautographs exposed 2 wk.

TABLE IV

Circulation Dynamics of Thoracic Duct Cells from Normal and Listeria-Infected Donors

Donor status*	Thoracic duct cells	Per cent lymphocytes labeled‡	
		Large and medium	Small
Normal	Donors	80.0	7.4
	Recipients§	<0.2	0.9, 0.9
Immune	Donors	88.8	17.8
	Recipients§	<0.2	0.6, 0.8

* Three normal (Lewis × DA)F_1 hybrid rats and three (Lewis × DA)F_1 hybrid rats infected with 9.3×10^6 *Listeria monocytogenes*. Animals injected every 8 hr for 6 days with 0.34 μCi of ^{3}H-thymidine per gram of body weight. First injection given at time of immunizing infection; lymph collected during first 24 hr after last injection.

‡ Radioautographs exposed 2 wk.

§ Two rats injected i.v. with 7×10^8 thoracic duct cells from labeled donors. Cells collected 24–36 hr after injection.

7×10^8 cells from *Listeria*-infected donors failed to inhibit the growth of *Listeria* in liver and spleen. By comparison, only 5×10^7 cells from the same *Listeria*-infected rats provided substantial protection in both organs. The results imply not only that the newly formed lymphocytes of infected rats fail to recirculate in large numbers from blood to lymph, but that the failure to recirculate is a property of the cells which convey antibacterial immunity.

Production of Recirculating Lymphocytes in Listeria-Infected Rats.—In the foregoing experiment, it was found that a few small lymphocytes generated during an acute *Listeria* infection were able to recirculate. It is possible that these join the mass of long-lived small lymphocytes that are generated throughout life at a rate proportional to body growth (12). It would be of interest to know whether the production of these cells is also influenced by infection. The delivery of newly formed, recirculating lymphocytes to the thoracic ducts of normal and *Listeria*-infected rats was therefore studied. For this purpose, the donor animals were injected repeatedly with ^{3}H-thymidine (Table IV). Thoracic duct cells, collected during the first 24 hr after the last of 18 ^{3}H-thymidine injections, were injected into syngeneic recipients, precautions again being taken to minimize reutilization of radioactive material released from labeled donor cells. Radioautographs prepared from the recipients' thoracic duct cells collected over a 12 hr period, 24–36 hr after injection, revealed that a portion of the small lymphocytes originally present in the donor inoculum had recycled from the blood (Table IV). But the number of labeled cells which followed this migration pathway was no greater in recipients of *Listeria*-immune cells than it was in animals given labeled thoracic duct cells from normal, nonimmunized donors.

DISCUSSION

Host resistance against *L. monocytogenes* rests ultimately with the macrophage. The fixed and mobile macrophages of *Listeria*-infected mice undergo remarkable changes in structure and function which are expressed in an ability of the cells to inactivate ingested organisms more rapidly (13). The process of macrophage activation has not been defined, although it is clearly driven by an immunological event in which specifically committed lymphoid cells have a crucial role (1). Results of the present investigation support this concept of a cell-mediated defense mechanism; they indicate that cells with the capacity to protect normal rats against listeriosis are lymphocytes, and that they are represented among the cells in the thoracic duct lymph of *Listeria-infected donors*.

The presence of the protective cells in central lymph identifies them as lymphocytes, but does not establish the species to which they belong. Rat thoracic duct lymph contains three categories of lymphocytes: a heterogeneous group of large and medium cells and two groups of small lymphocytes which cannot be distinguished in conventional histological preparations, but which differ with respect to turnover rate, and possibly also with respect to origin and immunological capacity (6). About 10% of the small lymphocytes normally present in the lymph have a rapid turnover rate, while the remaining 90% are produced more slowly (12).

Marked changes occur in the cellular makeup of thoracic duct lymph in

response to antigenic stimulation (3, 14). Large and medium lymphocytes, and small lymphocytes of the kind which have a rapid turnover rate, are released into the lymph in increased numbers. In the present investigation, it was found that the increase in newly formed lymphocytes is impressive during the first wk of a primary *Listeria* infection. On the 7th day, when the animal's thoracic duct cells are able to confer antimicrobial resistance upon normal recipients, the increase in newly formed small lymphocytes was approximately 10 times greater than the combined increase in large and medium lymphocytes.

The newly formed lymphocytes (large, medium, and small) which are added to the lymph of *Listeria*-infected rats include the specifically committed cells which convey antimicrobial resistance. It is presumed from studies in the mouse (2) that committed lymphocytes express their protective capacity through their ability to influence the microbicidal properties of macrophages. The ability of committed lymphocytes from the thoracic duct lymph of actively immunized donors to confer immediate protection against a challenge infection implies that the activation process is rapid and that the committed cells are functionally mature at the time of transfer.

A companion paper indicates that the lymph-borne cells which convey antimicrobial protection can move from the blood into an induced peritoneal exudate (15). Although it is not yet known what happens to them in the absence of an inflammatory stimulus, the current investigation indicates that they do not recycle to the thoracic duct in sufficient numbers to transmit immunity through an intermediate host. This failure of immunologically committed cells to recirculate places them in a class apart from the small lymphocytes normally resident in the circulating lymphocyte pool (9), and points to a physiological difference between the cells which initiate and those which express immunological reactivity. Thus, small lymphocytes which can initiate an immunological response such as the graft-*versus*-host reaction have a potentially long life-span, recirculate repeatedly from blood to lymph, and can realize their immunological capability after passage through an intermediate host (McGregor, D. D., unpublished observations).

Only a small fraction of the lymphocytes generated during a primary *Listeria* infection were able to circulate from blood to lymph. Their failure to transmit immunity to a second panel of normal recipients indicates that specifically committed lymphocytes do not readily recirculate. It must be admitted, however, that the stress of physical restraint and the high concentrations of thymidine and uridine used to inhibit the reutilization of radioactive nucleosides in the present experiments could have influenced the capacity of this cell type to recirculate.

Others have shown that recirculating lymphocytes are generated during the evolution of a graft-*versus*-host reaction (16) and the hemolysin response to sheep erythrocytes (17) in the mouse. It is not known, however, whether

these newly formed cells are the effectors of immunity, carriers of immunological memory, or whether they are antigen-sensitive cells that have a potential but as yet unexpressed immunological capacity. If antigen-sensitive cells are formed mainly in the thymus (6, 18) from which some antigens are largely excluded (19), their production might not be influenced by the antigenic stimulus provided by a systemic bacterial infection. The present observations provide some support for this notion by showing that the appearance of recirculating lymphocytes in the thoracic duct lymph of the rat is not significantly increased, and may even decline during a primary infection with *L. monocytogenes*.

SUMMARY

Thoracic duct cells from rats which have survived an infection with *Listeria monocytogenes* can confer a high level of antimicrobial resistance upon normal recipients. The cells which confer protection appear in the thoracic duct during the 1st wk of the immunizing infection, at a time when newly formed lymphocytes are being added to the lymph in substantially increased numbers. The protective cells differ in at least two respects from the majority of small lymphocytes in central lymph: they have a rapid turnover rate and a short life-span in the circulation.

Evidence was also obtained that lymphopoiesis affecting the long-lived small lymphocyte, which belongs to the recirculating pool, is not increased during an acute *Listeria* infection.

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