

MECHANISM OF GRAFT-VERSUS-HOST-INDUCED LYMPHADENOPATHY IN MICE

TRAPPING VS. PROLIFERATION*

BY EUGENE E. EMESON AND DONALD R. THURSH

(From the Department of Pathology, New York Medical College, New York 10029)

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The graft-vs.-host (GVH)¹ reaction has proved to be a very useful means of measuring the immunological reactivity of lymphoid cells to histocompatibility antigens. A variety of methods has been developed for quantitating this reaction. They include: (a) death or wasting syndromes in immunologically defenseless hosts (1-4), (b) the discriminate spleen assay (5), (c) splenomegaly in immunologically defenseless adult hosts (6), (d) chorioallantoic membrane (CAM) assay (7), (e) phagocytosis assay (8), (f) liver infiltration assay (9), (g) local lymphocyte infiltration assay in skin (10) or kidney (11) after local injection of donor cells, and (h) an in vitro assay (12). More recently, Ford et al. (13, 14) and Yoshida and Osmond (15) have developed a very sensitive GVH assay in rats, based on the observation of Levine (16) that when parental strain lymphoid cells are injected into the feet of F₁ hybrid hosts, the draining popliteal lymph nodes enlarge. This assay, with a number of modifications, has been adapted for use in mice by Hardt and Claesson (17). All of these methods, with the exception of the CAM assay and several studies specifically designed to measure donor cell proliferation directly (18, 19), measure GVH indirectly; i.e., they measure primarily the host's response to the GVH reaction.

In the most widely used of these assays, the discriminant spleen assay, the mechanism of splenomegaly has been extensively studied. Cytogenetic analyses of the proliferating cells in the enlarging host spleen clearly indicate that most of the proliferating cells are of host rather than donor origin (20). These findings are supported by studies in which previous irradiation of the host has been shown to inhibit the subsequent development of splenomegaly (21). The role of nondividing cells in GVH-induced splenomegaly, however, has not been investigated.

Recent evidence has indicated that a large part of the splenomegaly and lymphadenopathy resulting from ordinary antigenic stimulation is the result of the trapping of recirculating lymphocytes in the spleen and the lymph nodes, respectively (22-27). In the experiments to be described we have demonstrated that a major portion of the lymphadenopathy in the local GVH reaction in mice is similarly due to the trapping of recirculating lymphoid cells.

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¹ Abbreviations used in this paper: BAF₁, C57BL/6 × A/J F₁ mice; CAM, chorioallantoic membrane; GIBCO, Grand Island Biological Co.; GVH, graft-vs.-host; HBSS, Hanks' balanced salt solution; TC-199, tissue culture medium 199.

Materials and Methods

Experimental Design.—Graded doses of parental spleen or lymph node cells were injected into the right rear footpads of F₁ hybrid hosts. At 2–7-day intervals, the weights, the trapping of passively transferred ⁵¹Cr-labeled syngeneic spleen cells, and the uptake of [¹⁴C]thymidine by the right popliteal lymph nodes were determined. The left popliteal lymph nodes of the same mice (or other mice), challenged with a similar dose of syngeneic cells or with physiologic saline, were studied in the same way for comparison. Groups of four mice were employed for each cell dose in each experiment, and doses were graded in the ratio 125:25:5:1.

Animals.—Young, adult, female (6–12-wk-old) A/J mice were used as the donor strain, and C57BL/6 × A/J F₁ (BAF₁) female mice of a similar age were used as the host strain. They were purchased from the Jackson Laboratory, Bar Harbor, Maine.

Preparation of Cell Suspensions for Inoculation.—Suspensions of spleen and lymph node cells were prepared as previously described (27). In brief, they were minced in Hanks' balanced salt solution (HBSS) and passed through a series of graded stainless steel screens. After cell counts and standard trypan blue viability tests in Sahli hemacytometers, the cells were resuspended in a volume of HBSS so as to give 10.0×10^6 viable cells in a 20- μ l volume (20 μ l is the maximum volume that can be given intracutaneously into the rear footpad of a 20–25-g mouse). Two footpad injections, separated by 15 min, were required to get the maximum dose of 20.0×10^6 cells into the footpad.

Foot Injections.—The cell suspensions were taken up in a 100- μ l Hamilton syringe fitted with a 27-gauge needle. To inject the cells, the needle was inserted through the skin between the first and second digits and pushed into the loose subcutaneous space in the middle of the foot.

Labeling of Spleen Cells with ⁵¹Cr.—BAF₁ spleen cells were suspended at a concentration of 5.0×10^7 cells/ml in tissue culture medium 199 (TC-199) (Grand Island Biological Co. [GIBCO], Grand Island, N. Y.) containing 75 μ Ci/ml of sodium chromate ⁵¹Cr (Abbott Radio Pharmaceuticals, Chicago, Ill.), 15% IPT gamma globulin free fetal calf serum (GIBCO), 100 IU/ml of penicillin, and 100 μ g/ml of streptomycin, and incubated for 45 min in a humidified atmosphere of 5% CO₂ and 95% air. Excess ⁵¹Cr was removed by three washes with 50 ml of TC-199 containing 15% fetal calf serum. The cells were then passed through a 200-mesh stainless steel strainer, and 0.25-ml samples (containing 1.0×10^7 nucleated cells) were given intravenously to the hosts 24 h before sacrifice.

Radioactive Thymidine Incorporation in Lymph Nodes.—Host mice were given 5 μ Ci of [¹⁴C]thymidine intravenously and sacrificed exactly 2 h later.

Weights and Radiochemical Determinations.—After sacrifice, the host's popliteal lymph nodes were removed and carefully freed of fat. They were then washed and dehydrated with three daily changes of acetone, air-dried, weighed on a Mettler semimicrobalance (Mettler Instrument Corp., Princeton, N. J.) to an accuracy of 0.01 mg, and dissolved in 0.5 ml of N NaOH. Those labeled with [¹⁴C]thymidine were incorporated into a Cab-O-Sil cocktail (Packard Instrument Co., Inc., Downers Grove, Ill.) and counted in a Tri-Carb Liquid Scintillation Counter (Packard model 3375, Packard Instrument Co., Inc.), as previously described (27). Those labeled with ⁵¹Cr were counted in a well-type scintillation counter.

Analysis of Data.—The geometric means of the weights and counts per minute of the lymph nodes corresponding to each dose level were determined and plotted against the dose on double-log scales. When dose-response relationships were approximately linear, lines of best fit over that dose range were determined by the least square method (28). To test the hypothesis of linearity of regression, an analysis of variance technique comparing the variance within groups with the variance of the deviations of the group means from the estimated regression line was used (29). The hypothesis of linearity of regression was rejected when the calculated *F* ratio exceeded the table *F* value using an α of 0.05. Differences in the means of individual experiments were evaluated statistically by the Student's *t* test (30). In the experiments concerned with the trapping of ⁵¹Cr-labeled cells, the results of each day were normalized to

those of the other days by expressing them as a percentage of the counts per minute of a sample of the donor pool.

RESULTS

The data relating the weights of stimulated nodes to dose of donor cells are shown in Table I. The geometric mean weight of 20 normal unstimulated popliteal lymph nodes in 6–12-wk-old female BAF₁ mice was 0.21 mg \pm a standard error of the mean (SE) of 0.02 (range 0.10–0.30 mg). The mean weights of unstimulated popliteal nodes of mice 6 days after the injection of A/J spleen cells into the contralateral footpads showed a slight but statistically insignificant ($P > 0.01$) enlargement. 6 days after challenge by syngeneic BAF₁ cells, popliteal nodes also showed a slight degree of enlargement, which is statistically significant ($P < 0.01$) at a dose of 20.0×10^6 donor cells. With parental (A/J) cells, as in studies in rats (13–15), there is a linear dose-response relationship over a range of 0.8×10^6 – 20×10^6 for days 3–6 when plotted on a double-log scale. This is shown graphically for days 4 and 6 in Fig. 1. 6 days after the injection of the parental cells, the popliteal lymph nodes were seven to nine times heavier than the nodes of uninjected controls. In three additional experiments (data not given) in which a similar range of doses of donor cells was used, the linear dose-response relationships for days 3–6 were almost identical with those seen in Fig. 1.

Trapping of ⁵¹Cr-Labeled Lymphoid Cells.—The data relating the trapping of labeled BAF₁ lymphocytes in challenged lymph nodes during days 2, 4, and 6

TABLE I
Mean Weights (\pm SE) of Popliteal Lymph Nodes of BAF₁ Mice after Injection of A/J Spleen Cells, BAF₁ Spleen Cells, or No Cells into Rear Footpads

Day	Dose $\times 10^6$			
	0.16	0.8	4.0	20.0
		mg		
0	0.21 \pm 0.02 (20)*	—	—	—
2	0.26 \pm 0.01 (4)	0.35 \pm 0.02 (4)	0.28 \pm 0.02 (4)	0.45 \pm 0.08 (4)
3	0.28 \pm 0.07 (4)	0.24 \pm 0.04 (4)	0.35 \pm 0.13 (3)	0.82 \pm 0.09 (4)
4	0.31 \pm 0.04 (3)	0.28 \pm 0.08 (4)	0.61 \pm 0.07 (4)	1.30 \pm 0.06 (4)
5	0.25 \pm 0.06 (4)	0.37 \pm 0.01 (4)	0.78 \pm 0.35 (4)	1.26 \pm 0.13 (4)
6	0.27 \pm 0.07 (4)	0.38 \pm 0.12 (3)	0.73 \pm 0.10 (4)	1.76 \pm 0.09 (4)
6‡	0.22 \pm 0.03 (4)	0.24 \pm 0.05 (4)	0.32 \pm 0.07 (4)	0.31 \pm 0.04 (4)
6§	0.29 \pm 0.09 (4)	0.27 \pm 0.03 (4)	0.31 \pm 0.07 (4)	0.39 \pm 0.04 (4)
7	0.20 \pm 0.07 (4)	0.40 \pm 0.10 (4)	0.57 \pm 0.08 (4)	1.67 \pm 0.36 (4)

* Values in parentheses indicate the number of animals.

‡ Mean weights of unstimulated popliteal lymph nodes of BAF₁ mice 6 days after the injection of A/J (allogeneic) spleen cells into the contralateral footpads.

§ Mean weights of popliteal lymph nodes of BAF₁ mice 6 days after the injection of BAF₁ (syngeneic) spleen cells into the footpads.

to the dose of A/J donor cells are given in Table II. There was significant ($P < 0.01$) trapping of recirculating cells within 2 days of the initiation of the GVH reaction with the highest dose. Within 4 days, lymph nodes challenged with this same dose of A/J cells were trapping BAF₁ lymphocytes at seven times the normal rate. Trapping was still occurring on day 6, but at a somewhat reduced rate. These data are shown graphically in Fig. 2. The dose-response relationship is not linear when plotted individually for days 2, 4, and 6. However, when the total accumulation of trapped cells over the 6-day period is estimated, by normalizing and summing the accumulation of days 2, 4, and 6, the double-log plot is linear.

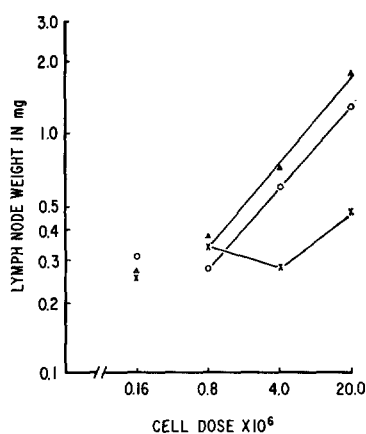


FIG. 1. Dose-response graph of the mean weights of the popliteal lymph nodes of BAF₁ mice 2 (X), 4 (O), and 6 (Δ) days after injection of A/J spleen cells into the rear footpads.

TABLE II

Mean Counts per Minute (\pm SE) of ⁵¹Cr-Labeled BAF₁ Spleen Cells Trapped in the Popliteal Lymph Nodes of BAF₁ Mice, 2, 4, and 6 Days after Injection of A/J Spleen Cells into Rear Footpads

Day	Doses $\times 10^6$			
	0.16	0.8	4.0	20.0
0	259 \pm 31 (22)*	—	—	—
2	297 \pm 10 (3)	349 \pm 89 (4)	340 \pm 137 (3)	990 \pm 193 (3)
4	355 \pm 45 (4)	541 \pm 47 (4)	1,543 \pm 144 (4)	1,982 \pm 78 (4)
6	297 \pm 83 (4)	753 \pm 146 (4)	830 \pm 30 (4)	1,269 \pm 283 (4)
Net‡	949	1,643	2,713	4,241

* Values in parentheses indicate the number of animals.

‡ Sum of the means of days 2, 4, and 6.

[^{14}C]Thymidine Uptake.—The data relating [^{14}C]thymidine uptake in counts per minute to the dose of parental cells 2, 4, and 6 days after the challenge are shown in Table III. In these experiments it was not possible to differentiate between the thymidine taken up by host and donor cells. There is a significant ($P < 0.01$) increase in thymidine uptake within 2 days of the initiation of the GVH reaction with the highest dose. This increase in thymidine uptake is 8–11 times that in unstimulated nodes at day 6. When these data are plotted on a double-log scale (Fig. 3), there is a linear response from 0.16 to 20.0×10^6 cells at day 6 and from 0.8 to 20.0×10^6 cells at day 4.

The times of onset of all three responses, i.e. increased weight, increased

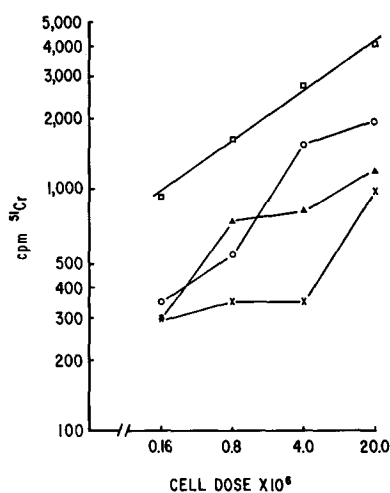


FIG. 2. Dose-response graph of the mean counts per minute of ^{51}Cr -labeled BAF_1 spleen cells trapped in the popliteal lymph nodes of BAF_1 mice 2 (\times), 4 (\circ), and 6 (Δ) days after injection of A/J spleen cells into rear footpads. Each point (\square) on the highest slope represents the sum of the geometric means of days 2, 4, and 6 (i.e., net accumulation). All mice received their respective portions of ^{51}Cr -labeled spleen cells 24 h before sacrifice.

TABLE III

Mean Counts per Minute ($\pm\text{SE}$) of ^{14}C in the Popliteal Lymph Nodes of BAF_1 Mice 2, 4, and 6 Days after Injection of A/J Spleen Cells into Rear Footpads

Day	Dose $\times 10^6$			
	0.16	0.8	4.0	20.0
0	193 \pm 15 (4)*	—	—	—
2	188 \pm 18 (4)	255 \pm 16 (4)	237 \pm 35 (4)	379 \pm 42 (4)
4	183 \pm 12 (3)	229 \pm 58 (4)	705 \pm 78 (4)	1,800 \pm 102 (4)
6	176 \pm 73 (3)	331 \pm 146 (4)	891 \pm 109 (3)	1,941 \pm 111 (3)

* Values in parentheses indicate the number of animals.

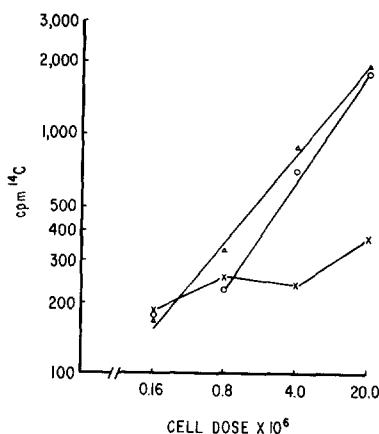


FIG. 3. Dose-response graph of the counts per minute of ¹⁴C in the popliteal lymph nodes of BAF₁ mice 2 (X), 4 (O), and 6 (Δ) days after injection of A/J spleen cells into rear footpads. 5 μCi of [¹⁴C]thymidine were given intravenously to each mouse 2 h before sacrifice.

trapping, and increased thymidine uptake, vary inversely with the dose of the parental cells used to initiate the reaction.

DISCUSSION

The present experiments clearly show that there is a selective trapping of ⁵¹Cr-labeled syngeneic lymphoid cells in lymph nodes subjected to a local GVH reaction. It is reasonable to assume that this is accompanied by a comparable selective trapping of the hosts' own unlabeled recirculating lymphocytes. The results of these experiments do not permit us to state the exact fraction of the observed lymph node enlargement that is related to trapping, although evidence that recirculating cells in transit make up a large part of the total cellularity of lymph nodes (31–33) makes it highly probable that trapping is a major factor. Proliferation of host cells within the reacting lymph node accounts for most of the remaining enlargement; proliferation of donor parental strain cells presumably contributes very little to the lymph node enlargement, inasmuch as very few donor cells reach the node (13).

Since the majority of the lymphocytes of the recirculating pool are long-lived (34), and since short-lived cells have little tendency to localize in lymph nodes (even with antigenic stimulation),² we assume that most of the lymphoid cells trapped during the GVH reaction are long-lived. The present studies do not provide any information as to whether these are long-lived T cells, B cells, or both (35).

² Emeson, E. E., D. R. Thursh, and T. Nobel. The partitioning of long- and short-lived lymphocytes in the lesions of cell-mediated hypersensitivity and their draining lymph nodes. Manuscript in preparation.

In lymph nodes subjected to conventional antigenic stimulation, most of the enlargement occurs within the first 24 h, a time period before there are substantial changes in lymph node DNA, RNA, or protein synthesis (36, 37). This suggests that most of the enlargement is due to lymphocyte trapping. There are at least two different mechanisms whereby this trapping of lymphocytes occurs. One is related to the immunological specificity of the lymphocytes being trapped (26, 27); the other is not (23, 26, 27, 38). The local GVH reaction seems to be an ideal model for the study of this latter mechanism because, with one possible exception (39) the F_1 host is totally lacking in cells that have specific immunological reactivity toward any of the parental strain antigens.

Although the trapping of specifically reactive lymphocytes can be projected as a mechanism for concentrating specific cells in a local lymph node to amplify the immune response, the significance of the trapping of cells lacking in specificity is not as readily inferred. It is tempting, however, to speculate that this nonspecific trapping of lymphocytes plays a role in the action of adjuvants (23, 24, 37), in the "allogeneic effect" described by Katz and Osborne (40, 41) and in the abrogation of tolerance to sheep erythrocytes by means of transferring allogeneic lymphocytes described by McCullagh (42). The studies of O'Toole and Davies (43) suggest that nonspecific trapping may be a factor in antigenic competition as well.

Finally, the present studies, along with those of Hardt and Claësson (17), have shown that the popliteal lymph node GVH assay described initially in the rat (13-15) can be used in the mouse and shows a similar cell dose dependency. Although the smaller size of mouse footpads and popliteal lymph nodes does present minor technical difficulties, they are balanced by the wider variety and greater availability of inbred strains of this species available for study.

SUMMARY

Graft-vs.-host (GVH)-induced lymphadenopathy of the popliteal lymph node has been produced in $C57BL/6 \times A/J F_1$ (BAF_1) mice by injecting A/J spleen cells into the rear footpads. By giving ^{51}Cr -labeled BAF_1 lymphoid cells intravenously to the hosts, 24 h before sacrifice, we have demonstrated that a large portion of the GVH-induced lymphadenopathy is due to the trapping of circulating lymphocytes in the challenged lymph nodes.

Most of the remaining enlargement can be attributed to proliferation of host cells within the reacting lymph nodes. Conditions have been defined under which the weights and [^{14}C]thymidine incorporation of the popliteal nodes can be plotted against the dose of injected A/J spleen cells on a double-log scale to give a linear dose-response. The popliteal lymph node GVH assay is a simple and effective means of quantitating immune reactivity to histocompatibility antigens in mice.

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