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Effect of Heparin Treatment on GVH Disease (38273)

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Heparin suppresses delayed hypersensitivity responses *in vivo*. After short-term administration, it depresses expression of ocular (1, 2), peritoneal (3), and skin reactions of cell-mediated immunity (4); after longer term administration, it inhibits development of experimental allergic encephalomyelitis (EAE) (5). The mechanism underlying these effects has been imperfectly characterized. We report here on GVH disease in heparinized mice and on the effect of heparinization on lymphocyte homing in GVH disease.

Materials and Methods. GVHR. Six- to eight-week-old A/Jax donor mice and 4-week-old (C57BL/6 × A)F_1 hybrid recipients were obtained from the Jackson Laboratory, Bar Harbor, Maine. Spleens from donor mice were teased gently in balanced salt solution (BSS). The cells obtained were washed twice, counted, checked for viability with trypan blue (> 95% viable), and injected ip or iv in 0.5 ml of BSS. Experimental recipients were injected ip with heparin (Liquahaemin Sodium 10, Organon, Inc.) or BSS morning and evening to sacrifice. Heparin doses varied from 2 × 15 to 2 × 40 U/mouse/day. Cells were given 1 hr after heparin. Control recipients were injected morning and evening with 0.5 ml BSS. The spleen/body weight ratio (i.e., mg spleen/g body wt) was calculated for each mouse using standard procedures (6) and mean values ± standard error of the mean determined for each group using Student's *t* test.

Prelabeling of long-lived donor lymphocytes with ^3H-thymidine. Groups of 3-week-old A/J mice were injected ip with ^3H-thymidine (thymidine-methyl ^3H, sp act 6.7 Ci/mmole, New England Nuclear), 0.5 μCi/g body wt. Each mouse received 12 injections over a 16-day period (7). Two weeks after the last injection the mice were killed and pools of donor cells prepared. GVHR was produced as described above. Recipients were sacrificed on the first, third, and fifth days after cell transfer and their spleens weighed. Three samples (10–15 mg each) from each spleen were next weighed, placed in individual scintillation bottles, dissolved in 1 ml of NCS tissue solubilizer (Amersham/Searle Corporation), and left overnight at 37°. After the tissue had dissolved, 16 ml of Omnifluor solution (Omnifluor, New England Nuclear—4 g/liter of toluene) was added and the samples left overnight in a dark room at 4°. Each vial was counted for 20 min in a Packard Automatic Tricarb Liquid Scintillation Counter and counts/min/sample determined after correction for background. The mean count/g of each of the three samples from each spleen was calculated, the results averaged and expressed as mean counts/min/g of spleen. Mean values and the standard error of the mean for each group of mice were determined. Each group contained a minimum of nine samples.

Results. Effect of heparin on lymphoid organs. Six hours after 50 U of ip heparin, the WBC count in six mice averaged 15,000 versus 8100 in five controls. After 2 weeks of continuous heparin (20 U twice daily), in contrast, the WBC had fallen to 3840 (five mice) versus 6850 in saline-injected controls (five mice). Thymus weights in saline-injected and heparin-treated controls were identical after 2 weeks of daily heparin. Heparin treatment for 8, 14, or 28 days led to a reproducible but statistically insignificant increase in spleen size as illustrated in Figs. 1 and 2.

Effect of heparin on GVH disease. Data obtained in mice given 20 U of heparin morning and evening are presented in Fig. 1. Heparinized mice had significantly smaller spleens ($P < .01$) on Day 8 than did control mice. Fifteen units twice daily failed to lessen the severity of GVH

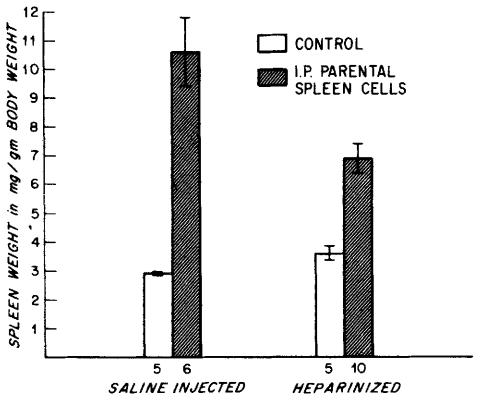

FIG. 1. Effect of 20 U heparin twice daily on spleen size in GVH mice 8 days after ip injection of 3.5×10^7 A cells into (C57BL/6 × A)F_1 hybrids. The number of mice per group is indicated under the abscissa.

reaction significantly, but 20, 30, and 40 U daily uniformly suppressed the GVH reaction (Table I). When the dose of cells was increased to 5×10^7 cells, the heparin effect was still apparent (Fig. 2). Mice in this last experiment were sacrificed at 28 days. Syngeneic cells at a dose of 5×10^7 cells/mouse were without effect on spleen size (Fig. 2). When cells from donors which had been heparinized for 3 days were given to untreated recipients, active GVH disease was produced which did not differ significantly (as judged by spleen size) from that observed with the same dose of cells from saline injected donors.

Effect of heparin on lymphocyte homing. The results of five experiments using different pools of donor lymphocytes are presented in Table II. After iv cell injection, a 30% reduction in spleen cell homing of labeled lymphocytes ($P < .05$) was apparent at 24 hr (Table II). Even 3–5 days after ip injection of labeled long-lived lymphocytes, a 60% reduction in splenic radioactivity in the heparinized groups ($P < .01$) was still apparent. Heparin doses of 15, 25, and 30 U twice daily all suppressed spleen homing. The absolute amount of splenic labeling varied from experiment to experiment but the labeling ratios of heparinized to control groups were comparable from one experiment to the next. Homing of cells into lymph nodes was also studied. Significantly less radioactivity was found in the nodes of heparinized mice than in the nodes of control mice 3 and 5 days after ip inoculation.

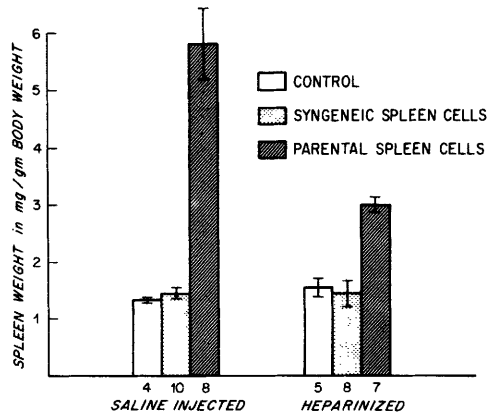

FIG. 2. Effect of 20 U heparin twice daily on spleen size 28 days after ip injection of 5×10^7 A cells on 5×10^7 (C57BL/6 × A)F_1 hybrid cells into (C57BL/6 × A)F_1 hybrids. The number of mice per group is indicated under the abscissa.

Discussion. The data presented show partial suppression of GVH disease, as measured by the spleen/body weight ratio (6), in heparinized recipient mice. This finding extends earlier work showing an effect of short-term heparin administration on expression of cell-mediated immune responses (1–4) and of longer term heparin administration on EAE, an autoallergic disease of cell-mediated immunity (5).

Decreased homing of labeled lymphocytes into the spleen after ip or iv administration of parent strain cells into F_1 hybrid recipient mice was also demonstrated. Since it is known that splenic homing of killed lymphocytes is minimal (8–10), and since substantial radioactivity was detected in the spleens of heparinized mice, a killing effect of the drug on the injected cells seems unlikely. Further, cells from donors which had been heparinized for 3 days showed full capacity to produce GVH disease when injected into normal recipients. This indicates a rapid restoration of normal capacity to produce GVH disease once heparin is removed, and also argues against a lethal effect of the drug, at the doses administered, on lymphocytes. The design of our experiments was such that escape from heparinization doubtless occurred overnight. Had heparinization been continuous, the differences between experimental and control groups might have been greater than were observed.

In earlier studies on the effect of heparin on the development of EAE, it was shown that the severity of EAE, as judged by clinical and histological criteria (5), is lessened by heparinization. During induction of EAE in rats, massive

TABLE 1. Effect of Various Doses of Heparin on GVH Disease in (C57Bl/6 × A)F₁ Hybrid Mice.

Cell dose	Heparin dose[a]	Spleen weight in mg/g body weight[b]		Significance[c]
		Heparinized	Saline injected	
3×10^7	15	8.2 ± .65 (4)	8.85 ± .54 (5)	.5
3×10^7	20	6.9 ± .42 (10)	10.6 ± 1.2 (6)	$< .01$
2.5×10^7	30	5.2 ± .15 (8)	7.2 ± .44 (12)	$< .05$
2.1×10^7	40	4.7 ± 1.0 (3)	7.7 ± .85 (4)	$< .1$

[a] Each mouse received the number of units indicated morning and evening beginning on day of cell administration. All mice were killed on Day 8.

[b] Mean weight ± SEM. Number of mice per group in parentheses.

[c] P values calculated according to Student's t test.

enlargement of the regional lymph node precedes development of disease. This enlargement depends, for the most part, on trapping of lymphocytes within the node (11), and is blocked by heparinization (12). Thus, heparinization impedes cell traffic into lymphoid organs in both EAE and GVH reactions, two distinct situations where cell traffic has a major role in determining the severity of the pathologic process.

How might heparin influence cell traffic? Surface properties appear to be important for transendothelial passage of lymphocytes since cells treated with enzymes which cleave surface components show impaired homing (13–15). Surface charge may be one factor influencing cell migration. Lymphocytes and endothelial cells carry negative surface charges, and the mutual repulsion between the two must be overcome when lymphocytes leave the blood. Activated lymphocytes carry less negative charge per unit of surface area than inactive cells (16), and there is evidence that they leave the blood more readily than resting lymphocytes (17). Heparin carries a large negative charge and interacts with the cell surface (18) increasing surface negativity. Thus, heparin, by increasing the mutually repelling negative charges on endothelial cells and lymphocytes, may impede cell migration. Such a thesis might explain why fewer cells enter inflammatory sites in heparin treated animals than in controls (19).

There is evidence that the morphologic features of vascular endothelium differ in spleen and lymph nodes. The pathways of cellular migration in the two organs differ as well (20, 21). Further, trypsin treatment impedes the ability of lymphocytes to enter lymph nodes, but not spleen, suggesting differences in cell migration mechanism in these two loci (15). It is of interest, therefore, that heparin interferes with cell migration at both sites.

The basis for the lymphocytosis in peripheral blood following short-term administration of heparin (10, 22–29) is not established. There is agreement that the lymph node cortex and the white pulp of the spleen are depleted of small

TABLE II. Effect of Heparin on Spleen Homing of ^{3}H-Thymidine-Labeled Long-Lived Lymphocytes Following Intravenous or Intraperitoneal Injection of A Cells into (C57BL / 6 × A)F₁ Hybrid Mice.

Route of cell administration	Cell dose × 10^7	Heparin dose[a](U)	Interval (days)	Counts/g of Spleen[b]		Percent Inhibition of homing	Significance[c]
				Heparinized	Saline Injected		
iv	2.5	30	1	7,479 ± 1,033	11,038 ± 1,250	32	$P < .05$
ip	2.5	25	3	722 ± 140	1,772 ± 210	59	$P < .01$
ip	2.5	30	3	1,159 ± 355	2,796 ± 446	63	$P < .01$
ip	3.0	15	5	519 ± 103	1,494 ± 256	65	$P < .01$
ip	2.5	30	5	1,986 ± 220	3,435 ± 383	42	$P < .01$

[a] Each mouse received dose indicated ip morning and evening beginning on the day of cell administration.

[b] Each value is an average of at least three mice ± SEM.

[c] Student's t test.

lymphocytes in heparinized animals (27, 29). In calves, increased numbers of lymphocytes appear rapidly in the thoracic duct following heparin treatment and lymphocytosis follows. Accordingly, an influx of cells from the lymphoid organs into the circulation under the influence of heparin has been postulated as responsible for heparin-induced lymphocytosis in this species (24, 28). In heparinized rats, in contrast, lymphocytosis without an increase in thoracic duct cell output is observed (10 but see 29), and labeled cells given intravenously show a decreased capacity to sequester in spleen and lymph nodes (10). Thus, lymphocytosis in this species may depend on decreased efflux of cells from the circulation. Further work will be required to determine which mechanism is responsible for heparin-induced lymphocytosis in mice.

Heparin in large doses is an antimitotic agent both *in vivo* (18, 30–32) and *in vitro* (18, 33) and suppressed proliferation of those cells which reach their target organs in heparinized mice is distinctly possible. The lymphopenia observed after 2 weeks of continued heparin is consistent with an *in vivo* antimitotic action of the drug. Lymphopenia after prolonged heparinization has been observed by one other group (28).

Heparin has been several times shown to impede the efficacy of killer cells *in vitro* (34–36). Recently, it has been suggested that benzyl alcohol, the preservative used in many heparin preparations, rather than heparin itself, is responsible for this effect (37). *In vivo,* on the other hand, suppression of cell-mediated immunity follows administration of depo-heparin which contains no preservative (5) and benzyl alcohol fails to provoke lymphocytosis (24).

Summary. GVHR induced by inoculation of parental (A/J) spleen cells into (C57BL/6 × A)F_1 hybrids is suppressed by heparin treatment of recipient mice, and splenic homing of prelabeled parental long-lived lymphocytes is reduced. Pretreatment of donor mice with heparin does not influence graft versus host reactivity.

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