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Postinflammatory Increase of Pathogenicity of Lymphocytic Inocula in the Peritoneal Cavity (42032)

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Abstract. Passive transfer of experimental allergic encephalomyelitis in rats by intraperitoneal injection of cells from draining lymph nodes was usually unsuccessful unless very large numbers of cells or conditioning procedures were employed. If the ip injection was done during the healing phase of a chemical peritonitis, then its effectiveness was greatly increased, even exceeding that of the iv route. The same effect of a healing chemical peritonitis was observed in adrenalectomized rats. A regional graft-versus-host disease produced by ip injection of parental spleen cells into hybrid recipients was greatly augmented in the healing phase of a chemical peritonitis. A host-versus-graft reaction against allogeneic spleen cells was histologically detectable only when the cells were injected after a chemical peritonitis. All these results are explicable as the consequence of a postinflammatory increase of absorption of lymphocytic inocula into the draining lymph nodes. The production of a chemical peritonitis is likely to be useful wherever immunologic and pathologic experimentation requires ip inoculations.

Immunization procedures, both active and passive, frequently make use of the peritoneal cavity as an access route to the draining regional lymph nodes. We discovered recently that absorption from the peritoneal cavity into the draining nodes of rats and mice was greatly increased during the healing phase of a local inflammatory reaction to a chemical irritant. Increased absorption of certain particulate and oily inocula was observed (S.L., submitted for publication). The present work concerns the increased absorption of cellular inocula from the peritoneal cavity. Three immunopathologic models were utilized: passive transfer of experimental allergic encephalomyelitis (EAE) with lymph node cells from actively immunized donors (1, 2); graft-versus-host (GVH) disease produced by injection of parental strain spleen cells into hybrid recipients (3); and host-versus-graft (HVG) reaction produced by injection of the host with foreign, antigenically dissimilar spleen cells. The success of our procedure with these as well as other types of inocula suggests that it will have wide applicability in experimental pathology and immunology.

Methods. Inbred isogeneic rats, 200–300 g, from Harlan Sprague–Dawley were maintained on Purina chow and tap water in hanging metal cages with wire mesh floors.

Chemical peritonitis was produced 1 week before the anticipated cell transfer procedures. This was usually done by a single intraperitoneal injection of sodium hypochlorite (household bleach, a 5.25% solution of NaOCl, diluted 1:100 in saline, dose of 50 ml/kg) or hydrogen peroxide (Fisher Scientific Co., type H322, a 3% solution of H_2O_2, diluted 1:30 in saline, dose of 150 ml/kg). The rats were fasted the night before this and other ip injections in order to reduce the incidence of inadvertent inoculations into the gastrointestinal tract. The efficacy of the treatment was verified at necropsy by macroscopic observations of perisplenitis, rounding of the liver edge, adhesions of upper abdominal organs, and shrinkage of the greater omentum. Microscopic study added surface fibrosis of omentum and peritoneal side of the diaphragm to this list.

EAE was induced in donor female Lewis rats by injection into one of the pads on the sole of the right foot of 0.05 ml of an emulsion of guinea pig spinal cord tissue in complete Freund's adjuvant (1, 2). Previously frozen cord tissue was thawed, strained, and homogenized in saline at 40% concentration, and then emulsified in an equal volume of adjuvant (Bayol F-Arlacel A, 85:15, with 4 mg/ml killed tubercle bacilli) and heated to

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60°C for 1 hr for this immunization. At the same time, 0.1 ml of a pertussis vaccine concentrate (20 billion organisms) was injected into the dorsum of the same foot for additional adjuvant effect. Six or seven days later, when most or all of the rats had not yet developed clinical signs of EAE, the draining lymph nodes were harvested (right popliteal, inguinal, axillary, renal, and all lumbar and pelvic nodes). A cell suspension in Hank's or Ringer's solution was prepared by cleaning and mincing the nodes, and straining and washing the suspension, all in the cold. Each recipient Lewis rat was given the yield from one donor (approximately 2×10^8 cells) or half that number, either ip without anesthesia or intravenously with the aid of ether for the penile vein injection or with cut-down for the external jugular vein injection in females. Starting 4 days later, the rats were checked daily for clinical signs of EAE: paralysis of tail (1+), weakness of hindlimbs (2+), paralysis of hindlimbs (3+). Eight days after passive transfer of EAE cells, the recipients were killed and the entire spinal cord was processed for histologic scoring of EAE lesions in hematoxylin–eosin-stained slides: four or five lesions in the entire slide (1+), a few lesions in a few fields (2+), many lesions in a few fields (3+), many lesions in many fields (4+). The severity of passively transferred EAE varies from time to time; therefore, every transfer included its own controls.

Adrenalectomy was done through a mid-dorsal skin incision. Rats were given saline as sole drinking fluid thereafter.

GVH disease was produced by ip injection of 2×10^8 spleen cells from normal Lewis rats into 240–290 g male Lewis XBN F_1 hybrid recipients (LBN) (3). The cell suspension was prepared in the same manner as EAE cell suspensions. The rats were killed 8, 11, or 14 days later, and the spleen and mediastinal nodes were weighed and processed for histologic study in hematoxylin–eosin-stained slides.

HVG reaction was produced by the reciprocal procedure, namely, LBN hybrid rat spleen cells were injected into 210–230 g male Lewis rat recipients. This choice of strains ensured the absence of any GVH component in the HVG reaction against the BN antigens in the inoculum. Procedures were the same as above except the recipient rats were killed after 7 days.

Results. *EAE.* Paterson and Fujinami (4, 5) have reported that passive transfer of EAE was only occasionally successful when approximately 2×10^8 cells (donor: recipient = 1:1) were injected ip, but a fourfold larger dose was capable of producing clinical signs and this has been our own experience as well. In contrast, iv injection of 2×10^8 immunized cells almost invariably resulted in signs of EAE in the recipient rats. Apparently many cells were detained or lost in the peritoneal cavity after ip injection. In view of our recent discovery that absorption of various inocula was increased during the healing phase of a chemical peritonitis, the ip route for passive transfer was investigated. Recipient rats were pretreated with various chemical irritants injected ip 1 week before passive transfer of EAE cells.

As anticipated, the control rats (saline pretreated) developed clinical signs of EAE in only one of eight passive transfers administered ip and summarized in Table I. In addition, histologic EAE lesions were absent or very mild in the control rats of the seven unsuccessful transfers. In contrast, recipient rats, either male or female, pretreated with NaOCl, H_2O_2, or a surfactant responded with clinical signs of EAE after 5, 6, or 7 days in each of the eight passive transfers by ip route. The severity of clinical signs and histologic lesions varied, but all eight transfers were more severe by both criteria in the rats with chemical peritonitis than in the single successful transfer in a control group. The chemical peritonitis was most successful when it was moderate in degree. Concentrations that were too high or too low, or the combined treatment with both NaOCl and H_2O_2 were less successful. Additional experiments, not recorded in Table I, showed timing of the pretreatment was critical. No enhancement was observed if the interval between NaOCl or H_2O_2 injection and EAE cells was 1 or 3 days instead of the usual 7 days.

Optimum procedures for ip transfer (derived from the data of Table I) were used in five additional transfers in order to compare their efficacy with passive transfers by iv route in the same experiments (Table II).

TABLE I. INTRAPERITONEAL TRANSFER OF EAE CELLS IS MORE EFFECTIVE AFTER CHEMICAL PERITONITIS

Intraperitoneal pretreatment		EAE clinical signs		EAE lesions[d]
Chemical[a]	(ml/kg)	Incidence[b]	Severity[c]	
NaOCl 0.05%	50	13/13	2.1	2.8
NaOCl 0.16%	50	2/3	1.0	2.3
NaOCl 0.05%	150	3/3	1.0	2.3
H_2O_2 0.1%	150	12/14	1.2	2.3
H_2O_2 0.3%	50	5/7	0.6	2.2
H_2O_2 0.75%	20	1/4	0.3	2.3
H_2O_2 0.05%	150	0/4	0	1.4
DOSS 0.01%	150	6/6	2.2	2.3
NaOCl + H_2O_2[e]		0/4	0	1.8
Silica 0.5%	2.5	0/4	0	0
Saline	50 or 150	2/29	0.02	0.3

[a] Injected ip 1 week before EAE cells (approximately $2/10^8$) injected by the same route. DOSS-dioctyl sodium sulfosuccinate (a surfactant).

[b] Numerator, number of rats with clinical signs (limp tail, weakness, paralysis). Denominator, total number of rats. Eight experiments are summarized; each included a control group of 3 or 4 rats.

[c] Average scores, scale of 0 to 3+.

[d] Average scores, scale of 0 to 4+.

[e] H_2O_2, 0.3%, 50 ml/kg, 9 days before EAE transfer, and NaOCl, 0.05%, 50 ml/kg, 7 days before transfer.

Two of these experiments utilized the same dose of cells as in Table I, and they revealed that EAE cells were as effective or more effective by ip route in pretreated rats as by iv route. The other three experiments utilized a lower dose of cells which clearly brought out the superiority of the ip route in pretreated rats over the iv route.

Experiments 1 and 4 in Table II also showed that pretreatment with H_2O_2 or NaOCl did not enhance the potency of EAE cells subsequently administered by iv route.

TABLE II. COMPARISON OF IP AND IV ROUTES FOR PASSIVE TRANSFER OF EAE

Expt No.	Intraperitoneal pretreatment[a]	EAE cells		EAE clinical signs		EAE lesions
		Route	Dose	Incidence	Severity	
1.	None	iv	2×10^8	3/4	1.0	3.4
	H_2O_2	iv	2×10^8	4/4	1.0	2.6
	None	ip	2×10^8	0/4	0.0	0.4
	H_2O_2	ip	2×10^8	4/4	1.0	2.3
2.	None	iv	2×10^8	5/8	0.9	ND[c]
	NaOCl	ip	2×10^8	8/8	2.5	ND
3.	None	iv	1×10^8	0/4	0.0	0.3
	None	ip	1×10^8	0/4	0.0	0.0
	NaOCl	ip	1×10^8	6/8	0.8	2.6
4.	NaOCl	iv	1×10^8	1/6	0.2	ND
	NaOCl	ip	1×10^8	7/8	1.8	ND
5.	None	iv	0.4×10^8[b]	0/4	0.0	0.0
	NaOCl	ip	0.4×10^8[b]	2/4	0.5	2.4

[a] H_2O_2, 0.1%, 150 ml/kg, or NaOCl, 0.05%, 50 ml/kg, 1 week before EAE cells.

[b] Unlike all other experiments, donor rats were not given pertussis vaccine, were used after 11 days, and only right popliteal nodes were harvested.

[c] Not done.

This observation proved that enhancement of EAE following chemical peritonitis was not due to a direct effect on the spinal cord because a direct effect on the cord would have been manifest regardless of the route by which the cells were injected. Experiments 1 and 3 in Table II included further control groups of ip EAE cells in rats without the benefit of pretreatment, and the absence of EAE was entirely in conformity with the control groups of Table I.

A chemical peritonitis might be expected to be stressful, and stress is known to inhibit EAE (6, 7), not to enhance it. Therefore, two groups of four rats were bilaterally adrenalectomized and 2 days later they were pretreated ip with saline or NaOCl. The adrenalectomized rats tolerated the NaOCl pretreatment quite well which indicated that it was not a very stressful procedure. When given EAE cells ip 1 week later, all four rats responded with EAE signs that were unusually precocious (onset after 4 or 5 days) and unusually severe (fatal within 1 day of onset). This result was consonant with the reported enhancing effects of adrenalectomy (2, 8). It shows that the effects of chemical peritonitis were independent of the adrenal gland and of adrenal-mediated stress effects. The four saline-pretreated controls, despite the expected enhancement of EAE by adrenalectomy, had no clinical signs and only minimal lesions, in agreement with the data of Tables I and II.

GVH disease. When spleen cells from Lewis rats were injected ip into LBN rats, all of the recipients developed GVH disease because of the reaction of the donor spleen cells to the foreign BN antigens on host cells. This was manifested as an enlargement of the mediastinal (and some abdominal) lymph nodes which received the lymphatic drainage from the peritoneal cavity. It was a regional GVH disease, analogous to the widely used popliteal lymph node assay following injections of parental spleen cells into the feet of hybrid recipients (3).

Control LBN recipients, pretreated only with saline and killed 8 or 11 days after Lewis spleen cell injection, had moderate enlargement of the nodes due to lymphoid, plasma cell, and epithelioid cell hyperplasia, characteristic of GVH disease. By 14 days, the node enlargement had receded and only a few, minor fibrotic foci remained as testimony to the previous bout of GVH disease (Table III). In contrast, the LBN rats pretreated with H_2O_2 had much more severe lymphadenopathy, more than double the weight of the controls at each time studied. In addition, the GVH disease showed no signs of recovery after 14 days, either by the size of the nodes or the histology.

It was necessary to exclude the possibility that the lymph node enlargement was a direct reaction to the H_2O_2 treatment. Therefore, additional rats were given ip injections of H_2O_2 without any subsequent GVH challenge. Their mediastinal lymph nodes were not significantly enlarged (average 0.09 g, SD $\pm$ 0.02, $N = 18$) compared to untreated controls (average 0.06 g, SD $\pm$ 0.03, $N = 15$). Additionally, none of these nodes had the characteristic histological appearance of GVH

TABLE III. REGIONAL GVH DISEASE AND HVG REACTION ARE INCREASED AFTER CHEMICAL PERITONITIS

Donor spleen cells	Recipient		Day of sacrifice (N)	Mediastinal lymph nodes (g)	Spleen (g)
	Strain	Pretreatment[a]			
Lewis	LBN	Saline	8 (7)	0.15 ± 0.08	0.70 ± 0.10
		Saline	11 (2)	0.16 ± 0.00	0.67 ± 0.03
		Saline	14 (5)	0.10 ± 0.01	0.58 ± 0.04
		H_2O_2	8 (3)	0.37 ± 0.09	0.98 ± 0.14
		H_2O_2	11 (2)	0.41 ± 0.14	1.18 ± 0.46
		H_2O_2	14 (5)	0.31 ± 0.17	1.01 ± 0.30
LBN	Lewis	Saline	7 (4)	0.06 ± 0.01	0.49 ± 0.04
		H_2O_2	7 (4)	0.09 ± 0.02	0.54 ± 0.03

[a] Saline or 0.1% H_2O_2, 150 ml/kg, ip, 7 days before spleen cells were injected ip.

disease as seen in the rats that received spleen cells.

Even a regional GVH reaction eventually reaches the spleen (3). This occurred in these experiments also, but significant splenomegaly (Table III) and histologic evidence of GVH disease in the spleen developed only in the H_2O_2 pretreated rats. The changes observed in the mediastinal nodes and spleen could be due to a postinflammatory increase of absorption of Lewis spleen cells from the peritoneal cavity into the draining lymph nodes and eventually into the blood stream. However, splenomegaly could also be due to enhanced absorption directly into the blood stream.

HVG reaction. When spleen cells from LBN rats were injected ip into Lewis recipients, they were subject to rejection, like any allogeneic graft. The mediastinal lymph nodes of the rats pretreated with H_2O_2 were only slightly larger than the nodes of the controls (Table III). Histologically, a few large, prominent germinal centers were present in nodes from both groups, but the rats pretreated with H_2O_2 also had striking expansion of the medullary cords filled with plasma cells. This was not due to the H_2O_2 pretreatment as it was not present in four other experiments in which nodes from H_2O_2 pretreated rats were compared to saline controls. It probably was due to an HVG reaction to foreign spleen cells absorbed in greater than normal amount.

Discussion. We have presented evidence to indicate that increased lymphatic absorption in the healing phase of a chemical peritonitis was due to loss of the sequestering and scavenging functions of the greater omentum, perhaps aided by an increase in the number and permeability of the diaphragmatic lymphatic vessels (S. L., submitted for publication). In that work, we had the advantage of using a particulate dye, a metal, and an oil, all of which were readily visible macroscopically and microscopically in increased amounts in draining lymph nodes. In the present work, there is no doubt that there was a postinflammatory enhancement of absorption, but the route of that absorption has not been so directly demonstrated.

In the case of GVH disease, the location of the lymphadenopathy in the mediastinal and abdominal nodes made it clear that the injected donor cells had passed into the diaphragmatic lymphatics even if it is understood that host as well donor cells contribute to the enlargement. Besides, the large literature on the regional GVH disease in the popliteal node which followed our original description (3), attests to the importance of lymphatic pathways. Similarly, the medullary plasma cell response in mediastinal nodes in the HVG reaction is reasonably attributed to lymphatic absorption of injected cells even if the observed changes are due entirely to the host's reaction to foreign antigens.

In the EAE experiments, the increased pathogenicity of the injected cells was compatible with an increase of absorption, but the route of absorption has not been demonstrated directly or indirectly. Our assumption that the route was lymphatic will have to rest on analogy with all the other inocula, and the fact that the lymphatic stream is the usual channel for absorption of cellular inocula. This assumption is fortified by the fact that EAE cells are known to take up residence in the lymphoid tissues even when they are injected by iv route (9).

If a sufficient number of cells are injected, EAE can be produced in rats by inoculation into the unmodified peritoneal cavity (4, 5). EAE cells that have been activated *in vitro* have also been effective by ip route in rats and other species (10–12). Yet, the ip route, as ordinarily used in rats, is clearly inferior to the iv route. In the postinflammatory state, especially after the use of NaOCl, however, the ip route was superior to the iv route (Table II). Two possible explanations can be suggested. The slower absorption of the EAE cells after ip inoculation may permit some further development of encephalitogenicity due to residual mitogenic or antigenic stimulation that remained with the inoculum despite the dissociation and washing procedures. No such opportunity would be present after iv injection because of the almost immediate isolation of each EAE cell. It is even conceivable that the chemicals used for pretreatment were themselves mitogenic. However, NaOCl and H_2O_2 are highly reactive chemical species and we have evidence that their enhancing activity is exhausted within 5 min of injection (S.L., submitted for publication). Therefore, interaction with cells injected 1 week later is extremely unlikely.

Alternatively, the iv route may be inherently inefficient because of an immediate and permanent sequestration and destruction of part of the inoculum in lungs or liver. Cells absorbed from the peritoneal cavity would pass through the nodes first, and are likely to enter the blood stream individually, hence less chance to be trapped while passing through pulmonary capillaries or liver sinusoids. Presumably, neither of these explanations apply to the unmodified peritoneal cavity because the absorption of cells is so low that other factors are insignificant.

We have demonstrated a postinflammatory increase of lymphatic absorption with respect to all types of inocula; therefore, prior production of a chemical peritonitis may be useful in many areas of experimental pathology. Active immunization with cellular, particulate and soluble antigens is likely to prove a fertile field for application of this method. Important results have already been obtained in EAE produced by active immunization (to be published).

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