Role of the Central Nervous System in Acute-Phase Responses to Leukocytic Pyrogen

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Intracerebroventricular injection of rabbit leukocytic pyrogen (LP) into conscious, healthy cannulated rabbits produced markedly enhanced febrile and acute-phase responses as compared with equivalent-dose, single bolus intravenous injection. The increased effectiveness in inducing granulocytosis and hypoferrinemia on intracerebroventricular injection was matched by changing the method of administration of intravenous LP from a single initial bolus to multiple fractional doses over a 2-h period. This suggested that augmentation for these parameters may have reflected only a "reservoir" function of the cerebral ventricles which prevented rapid clearance of LP from the blood. The ability of LP to induce hepatic synthesis of haptoglobin and C-reactive protein was so markedly enhanced by intracerebroventricular injection, however, that a role of the central nervous system in mediating or in modifying in an important way a non-neural mechanism for this mediation must be postulated.

During inflammatory states, activated granulocytes, monocytes, and macrophages of humans and most higher mammalian species can release a small neutral protein, leukocytic pyrogen (LP), which acts on specific receptors in the preoptic area of the anterior hypothalamus to initiate fever (2, 7). Recently, other important physiological activities of, or intimately associated with, the LP molecule have been demonstrated. Rabbit LP has been shown to induce the whole sequence of acute-phase changes in recipient rabbits or rats. Rabbits respond to a single intravenous (i.v.) injection of LP with a characteristic fever (0 to 3 h after injection), with marked polymorphonuclear leukocytosis and a fall in serum concentrations of iron (2 to 8 h after injection) and with typical changes in the concentration of plasma proteins (12 to 36 h after injection): C-reactive protein (CRP) appears in significant amounts; fibrinogen, haptoglobin, ceruloplasmin, alpha 1 acid glycoprotein, and other rabbit acute-phase reactants increase significantly, whereas transferrin and albumin concentrations fall (5, 28). The acute-phase responses in rats to intraperitoneal injections of rabbit LP have been studied extensively. Although rats do not become febrile in response to these heterologous LP preparations so administered, they show granulocytosis, hypoferrinemia and hypozincemia, amino acid fluxes to the liver, increased ribosomal ribonucleic acid synthesis in the liver, induced pancreatic secretion of glucagon, and analogous induced changes in hepatic protein synthesis and in plasma protein concentrations (11, 15, 18-19, 33-34). These phlogistic

activities of LP have been referred to as leukocytic endogenous mediator (LEM) activity (13, 31). More recently, another important and related activity, the activation of human peripheral blood granulocytes by partially purified human mononuclear pyrogen, has been demonstrated by Klempner et al. (21, 22).

The pyrogenic and the phlogistic activities of LP/LEM have not been dissociable as yet. Bornstein and Walsh found that all the acute-phase stimulating activity in LP preparations remained coherent and associated with the pyrogenic activity after gel filtration chromatography (5). Kampschmidt and co-workers (29) purified rabbit LP extensively by the methods of Murphy et al. (30) and recovered all the LEM activity in the purified LP fraction. Although one study claimed to have separated pyrogenicity from LEM activity (27), the methods employed were indirect and not well quantified and involved partial differential destruction rather than recovery of two or more separable active materials. Subsequent studies by Kampschmidt further documented the strict parallelism of LP and LEM activities in rabbit LP preparations (17). In studies of partially purified human monocytic pyrogen in the rat and rabbit, pyrogenicity and LEM effects were also not dissociable (16), and the extent of *in vitro* stimulation of human blood granulocytes by human monocytic pyrogen was found to be directly proportional to its pyrogenicity in the rabbit (21).

The pathways of mediation of many of the acute-phase responses to LP are as yet unclear. In early *in vitro* studies, direct incubation of LP

with liver slices did not lead to the anticipated alterations in protein synthesis (14). A more complex interaction involving humoral, endocrine, and possibly neural systems must be involved in the many physiological changes induced by LP.

In 1976, Bailey et al. described augmented LEM effects in rats in response to intracerebroventricular (ICV) injections of rabbit LP as compared with the customary intraperitoneal injection and suggested that the central nervous system (CNS) might be a primary site of action for the LEM effects as well as for the pyrogenicity of LP (3). This suggestion seemed at variance with several known effects of LP/LEM which appear to be direct peripheral actions, such as the induced hypoferrinemia, which is thought to be secondary to the release of lactoferrin from circulating neutrophils by LP (21, 32). It seemed necessary to confirm or reject the thesis of a central role for the CNS if we wished to understand the site and mode of action of LP/LEM.

We set out to re-examine the effects of ICV injections of LP in the rabbit by using more quantitative methods and by employing special precautions and sensitive *Limulus* amoebocyte lysate testing to ensure endotoxin-free conditions. A dose-response curve for the phlogistic activity for graded i.v. doses of LP in the rabbit was required, analogous to earlier dose-response curves of pyrogenicity (4), to evaluate responses to ICV injections more quantitatively.

Our data confirm significantly increased responses to single ICV injections compared with equivalent single i.v. doses. Some of this augmentation may be related to a reservoir function of the cerebral ventricles which prevents the rapid clearance of LP that occurs after i.v. injection. However, the LP-induced hepatic protein synthesis of acute-phase proteins is so increased as to suggest a possible important role of the CNS in this aspect of LP/LEM action.

MATERIALS AND METHODS

Endotoxin-free conditions and preparation of crude LP. Methods for maintaining glassware and solutions free of bacterial endotoxin, induction of glycogen-saline peritonitis, processing of exudates to prepare crude leukocytic pyrogen, and pyrogen testing in trained rabbits have been described in previous reports (5). Crude LP, the saline extract of 70×10^6 exudate-derived granulocytes per ml, contains 70×10^6 cell equivalents (70 MCE) of LP per ml. An 0.25-ml aliquot (17.5 MCE) of crude LP (100 to 125 μ g of protein) is a minimal pyrogenic dose, i.e., that amount which elicits a clear monophasic LP fever response of $0.7^\circ\text{C} \pm 0.2^\circ\text{C}$ at 45 min after an i.v. injection in healthy trained rabbit recipients. Crude LP is very stable at 4°C and is comparable in protein, pyrogenicity, and LEM activity from preparation to preparation in our hands. LP preparations are almost invariably sterile and free

of endotoxin at the 0.06-ng/ml threshold of our test system. Only sterile and endotoxin-free preparations are used.

***Limulus* assay.** *Limulus* amoebocyte lysate (Associates of Cape Cod, Woods Hole, Mass.) was used to test for endotoxin contamination, as described previously (5).

Peripheral injection of LP and collection of blood. A series of 8 to 18 healthy, white, trained male rabbit recipients was injected via the marginal ear vein with aliquots of rabbit LP derived from 17.5×10^6 to 350×10^6 leukocytes (17.5 to 350 MCE). Blood samples were obtained from the marginal veins of the warmed ear at 0, 8, 24, and 48 h after injection. Leukocyte counts (Coulter Counter) were obtained, and smears for differential counts were prepared within 1 h of collection.

Analysis of acute-phase reactants. Total serum iron concentrations were determined by the ferrozine technique (8). Haptoglobin and CRP concentrations were determined by quantitative radial immunodiffusion with antisera prepared in our laboratory (6, 26).

ICV injections. With an aseptic technique, a sterile Kopf cannula (model 201, Kopf Instrument, Tujunga, Calif.) was inserted into the lateral ventricle of lightly anesthetized New Zealand white male rabbits entering 2.5 mm from the midline at the bregma with a 9-mm cannula. The animal was observed for 7 to 10 days before pyrogen testing. During such time and for periods up to and over 1 year, the animals remained afebrile, free of infection, and steadily gained weight. Placement of the cannula within the ventricular system was confirmed by X-ray after injection of air or contrast material and by dissection postmortem. The maximum volume injected through the cannula in any experiment was 0.25 ml. The cannula was flushed with an equal volume of pyrogen-free saline through the neoprene diaphragm after the LP injections.

Controls. Changes seen with LP were compared with changes seen with injections of pyrogen-free saline and LP heated to 90°C for 30 min. When possible, the same rabbits were used for i.v. and ICV injection.

Multidose injections. A minimal pyrogenic dose (17.5 MCE) was divided into six equal portions (2.9 MCE) which were given i.v. at 20-min intervals for 100 min. Temperature responses were monitored for the first 3 h after injection by using rectal thermistor probes and a 24-channel recorder.

Experimental animal conditions. The animal care facilities of the Syracuse Veterans Administration Hospital observe the Public Health Service and National Research Council recommendations for animal care. White male, 2.5- to 3-kg New Zealand rabbits were used for all experiments. No animals manifesting any clinical illness, base-line temperature over 39.8°C , or base-line CRP concentration greater than 10 $\mu\text{g}/\text{ml}$ were used for experimentation.

Statistical analysis. The response to injected fractions was analyzed by the paired *t* test for mean differences of self-paired samples, comparing the values of serum samples obtained before injection and at peak response times (8 h for serum iron and leukocyte counts and 24 h for CRP and haptoglobin) for each recipient. The *P* values of the mean differences over time were obtained from standard tables from the calculated *t* value.

RESULTS

Acute-phase responses to graded i.v. doses of LP. In Fig. 1 the mean changes from base line in blood granulocyte counts (A) and in serum iron concentration (B) 8 h after a single i.v. dose of rabbit LP are displayed. The LP doses are plotted on a logarithmic scale. The

FIG. 1. Granulocytosis (A) and fall in serum iron (B) at 8 h after single i.v. injections of LP/LEM. Each point represents the mean response of 6 to 18 rabbits. Bars depict the range $\pm$ standard error of the mean. Control injections of i.v. saline produced a fall in granulocyte count of $0.25 (\pm 0.13) \times 10^3$ and an increase in serum iron of $11.7 (\pm 4.5) \mu\text{g}/100 \text{ ml}$ at 8 h in seven rabbits. The responses to all doses of LP tested are significantly different from saline controls and from heated LP controls ($P < 0.01$). The mean values (± 1 standard deviation) for total leukocyte count and total granulocyte count in 75 untreated rabbits were $8.45 (\pm 1.86) \times 10^3$ and $2.96 (\pm 1.27) \times 10^3$, respectively. The mean value for serum iron (± 1 standard deviation) in 48 untreated rabbits was $218 (\pm 74) \mu\text{g}/100 \text{ ml}$.

marked granulocytosis induced by LP appeared to plateau at doses above 70 MCE, reminiscent of the febrile responses to i.v. doses of LP in this dose range (4). The fall in serum iron at 8 h and the increased serum concentrations of haptoglobin and CRP at 24 h (Fig. 2) show characteristic log dose responses for the range of LP doses employed. There is variability in base-line haptoglobin concentration between individual rabbits, but the range of induced responses is similar between rabbits and is reproducible for each rabbit. Saline and heated LP controls produced no significant changes in any of these parameters at 8 or 24 h.

Acute-phase responses to ICV injections of LP. With this data base for dose responses to LP given i.v., we next examined the effects of ICV injections of aliquots of LP in conscious, chronically cannulated rabbits. Injections of 1.75 MCE, a subfebrile dose of LP by i.v. injection, consistently produced temperature elevations of 1.7°C and above in recipients when given through the CNS cannula. Fever persisted for 4 to 6 h, a longer period than that for a single pyrogenic dose of LP given i.v. A dose of 17.5 MCE ICV gave somewhat higher and more protracted fevers. Injections of saline or heat-inactivated LP into the cannula produced neither significant fever elevation nor acute-phase changes. The effects of ICV injections of 1.75

FIG. 2. Increases in serum concentrations of CRP (left) and haptoglobin (right) at 24 h after single i.v. injections of LP. Each point represents the mean response of 8 to 14 rabbits, except CRP response to 17.5 MCE (five rabbits). Bars depict the range $\pm$ standard error of the mean. Saline injections produced a fall of CRP of $0.5 \mu\text{g}/\text{ml}$ (six rabbits) and a fall in serum haptoglobin of $3.5 (\pm 1.3) \text{ mg}/100 \text{ ml}$ at 24 h in six rabbits. The responses to 35 MCE and above are significantly different from saline controls and heated LP controls at a P value of < 0.01 . The mean value of serum haptoglobin (± 1 standard deviation) in 99 untreated rabbits was $31.3 (\pm 14.6) \text{ mg}/100 \text{ ml}$.

and 17.5 MCE on circulating granulocyte counts, serum iron, haptoglobin and CRP are recorded in Table 1. Both 1.75 and 17.5 MCE, doses which produced minimal acute-phase changes when given i.v., produced clear-cut changes in all parameters tested when introduced into the cerebral ventricles. Induced granulocytic leukocytosis and hypoferrremia were only moderately augmented over responses to equivalent amounts of LP given i.v., approximately 2- to 10-fold, but haptoglobin and CRP elevations were strikingly augmented (approximately 50 to 100 times and 100 to 300 times, respectively), suggesting a dichotomous mode of enhancement of the LEM effects.

Acute-phase responses to multiple i.v. doses. Leukocytic pyrogen, a small protein of about 13,000 daltons, is rapidly cleared from the peripheral blood. The enhanced effect of ICV injection might therefore be due in part to a reservoir or depot effect in which LP is released more slowly into the circulation and avoids the usual rapid clearance of an i.v. bolus. To test this hypothesis, a dose of 17.5 MCE was divided into six equal parts and administered i.v. at 20-min intervals (Table 1). This method of administration increased the effectiveness of LP markedly in terms of granulocytosis, hypoferrremia, and induced CRP and haptoglobin synthesis (approximately 8 to 20 times), matching the effect of ICV injection for the first two parameters. ICV injection, however, induced changes in acute-phase protein synthesis that were considerably above and beyond those explainable by a simple reservoir function. A dose of 17.5 MCE of LP given ICV resulted in elevations of CRP and haptoglobin concentrations far in excess of elevations induced by 17.5 MCE given in divided doses over 2 h or by 175 MCE given as a single dose i.v.

DISCUSSION

There is at present no satisfactory unifying mechanism of action which can encompass the

many biological activities of LP. Fever appears to be explained by a direct action of LP on receptors in the preoptic area of the anterior hypothalamus because it can be induced by direct inoculation of minute amounts of purified LP into this specific site (9). Activation of blood granulocytes to increase oxidative metabolism and to release lactoferrin appears to be a direct effect of LP, since it can be demonstrated by addition of highly purified LP to blood leukocytes in the absence of plasma (21). Certain of the observed effects appear to be secondary phenomena, such as the fall in serum iron, which is presumed to be secondary to the direct effect on granulocytes with the accompanying release of lactoferrin, and the rise in serum copper, which shows that increased synthesis of ceruloplasmin has occurred.

There is inadequate data as yet to explain the site and nature of the very selective and complex stimulation of hepatic protein synthesis which produces the acute-phase protein responses, the induced leukocytosis, the induced pancreatic secretion, or the induced amino acid fluxes.

The possibility that the CNS might participate in the mediation of some of the observed acute-phase changes is raised by the fact that the pyrogenic response to LP is centrally mediated and by some prior observations that LP injected into the cisternal space or the lateral ventricles produced markedly enhanced fevers and elicited with heightened sensitivity some of the other phlogistic responses seen with i.v. or intraperitoneal administration (1, 3, 20). Bailey et al. (3) found an apparent enhanced responsiveness by injection of rabbit LP into the lateral cerebral ventricles of rats. They noted depressions of serum iron and zinc, elevation of serum copper, amino acid fluxes from plasma to the liver, and induced synthesis of an acute-phase reactant, alpha 2 macrofetoprotein, in response to doses of 5 MCE of rabbit LP, doses which were not effective by intraperitoneal injection. From these data, they postulated a CNS role in

TABLE 1. Comparison of responses to i.v. and ICV injections of rabbit LP

Dose of LP (MCE)	Route	No. of recipients	Increase in granulocytes ($\times 10^3$)	Fall in serum iron ($\mu\text{g}/100\text{ ml}$)	Increase in haptoglobin ($\text{mg}/100\text{ ml}$)	Increase in CRP ($\mu\text{g}/\text{ml}$)	Peak Δ temp ($^{\circ}\text{C}$)
1.75	ICV	10	2.5 (0.52) ^a	11 (4.6)	30.0 (5.3) ^b	24.9 (3.4) ^b	1.7-2.1 ^b
17.5	i.v.	5-18	2.6 (0.42)	30 (7.2)	5.7 (2.1)	1.4 (1.2)	0.7 $\pm$ 0.2
17.5	i.v.-D ^c	10	8.5 (1.0) ^b	125 (19.9) ^b	20.4 (2.8) ^b	9.5 (2.4) ^b	1.2-1.5 ^b
17.5	ICV	8	3.6 (0.64)	99 (14.3) ^b	59.8 (8.3) ^b	50.4 (4.4) ^b	2.4-2.8 ^b
175	i.v.	6-10	7.3 (1.4) ^b	84 (12.1) ^b	30.8 (5.6) ^b	9.6 (3.2) ^b	1.25 $\pm$ 0.25 ^b
17.5							
Heated	ICV	4	-0.4 (0.18)	4 ($\pm$ 2.9)	5.5 ($\pm$ 2.7)	-0.5 ($\pm$ 0.3)	<0.3
Saline	ICV	4	-0.2	+37 ($\pm$ 11.8)	-7.7 ($\pm$ 3.2)	0.5	<0.3

^a Mean values; parentheses indicate standard error of the mean.

^b Significantly ($P < 0.01$) greater than the response to a single i.v. dose of 17.5 MCE.

^c Divided doses: 2.9 MCE every 20 min $\times$ 6.

the mediation of these acute-phase responses. Because the authors did not establish the dose of LP required by the i.v. or intraperitoneal route to achieve equivalent phlogistic responses, it is not clear whether they had observed a 2-fold or a 50-fold enhancement by ICV injection.

Our studies were carried out in the rabbit. To be able to interpret relative sensitivities to i.v. or ICV injection, we needed to establish dose-response curves for the parameters under study. We were then able to show an increase in sensitivity for all five parameters by ICV injection as compared with i.v. injection and to discern that although the effect on hypoferrremia and on granulocytosis was modest and could be explained by delaying clearance of LP from the bloodstream, the effect on induced hepatic synthesis of acute-phase proteins was major. Our findings strongly suggest a role of the CNS in the mediation of the specific induced alterations in hepatic protein synthesis that characterize the acute-phase reaction.

We have no data about the pathway of this mediation, whether neural, endocrine, or neurohumoral. There is some fragmentary evidence to suggest that sympathetic pathways could be involved. The liver is richly supplied with adrenergic and parasympathetic fibers (23-24). Kushner has examined rabbit liver tissue, sampled at various time intervals after an inflammatory stimulus, to determine the sites at which the induced acute-phase synthesis is initiated (25). In his studies CRP appeared first in cells at the periphery of the liver lobules. In anatomic studies, this is also the site at which sympathetic nerve fibers enter the liver (10). Although studies with α and β adrenergic blockers could be employed to test this hypothesis, such studies might not be definitive. Sympathetic denervation of the liver is difficult and would probably require nothing less than complete removal and immediate reimplantation of the liver in situ, since sympathetic fibers enter along all the blood vessels. Hepatic autotransplantation in situ requires microsurgery to reanastomose all the severed vessels, but this should assure denervation for a period of time long enough to demonstrate return of good hepatic function and to carry out the necessary experiments. Transection of sympathetic outflow tracts in the cervical cord and demonstration of complete denervation would be another short-term approach but carries with it the risk of infection and inflammatory changes in a paralyzed laboratory animal.

Another major question as yet unresolved is whether the observed CNS effects represent the primary mediation of LP action on hepatic synthesis or whether the CNS role is only one of nonspecific stimulation. In the former case, the

CNS message would contain all the specificity required for the more or less coordinated pattern of changes seen during acute-phase responses, and all LP effects on acute-phase proteins would flow from receptors in the CNS. In the latter case, hepatic receptors for LP (or for some secondary humoral mediator elicited by LP) would initiate the pattern of selective protein synthesis, but the degree of stimulation could be increased by some permissive CNS intervention, such as increased rate of amino acid transfer to the liver. Since adrenergic mechanisms have been implicated in the secretion of glucagon (12), since glucagon secretion has been noted in response to LP/LEM injection (11), and since adrenergic mechanisms are the effector arm of the febrile response to LP, neural effects on hepatic protein synthesis may well be mediated via the hepatic sympathetic innervation.

The CNS effects that we have observed raise further questions not only about the mechanisms of LP action but also about the role of the CNS in the control or regulation of other aspects of protein synthesis and of other metabolic pathways.

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