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The Immune Response Evokes Changes in Brain Noradrenergic Neurons

Abstract. *A decreased noradrenaline turnover in the hypothalami of rats was observed at the peak of the immune response to sheep red blood cells. The decrease in noradrenergic neuronal activity was mimicked by injection of soluble mediators released by immunological cells activated in vitro. Noradrenaline also tended to decrease in the brainstem but not in the residual brain. It is suggested that products released from activated immunological cells during the immune response may induce the previously described autonomic and endocrine mechanisms that contribute to immunoregulation.*

The immune response is a phasic phenomenon that includes well-programmed interactions among different cell types and molecules. Signals, originating from cellular and molecular elements of the immune system itself, constitute a level of autoregulation. There is also evidence of another more integrative level of regulation mediated by neuroendocrine signals (*1*). That there are information channels between immunological cells and the central nervous system (CNS) is indicated by the finding that changes in firing rates of neurons of the medial hypothalamus occur during the immune response (*2*). Since aminergic neurons projecting to the hypothalamus are important controllers of hypothalamic neuronal activity, we have considered the possibility that immunological signals could affect aminergic circuits within the CNS, which in turn could mediate the noradrenaline (NA) changes in lymphoid organs (*3*) and the hormonal changes (*1*) observed during the immune response. We report here that the immune response elicits a decrease in NA synthesis in the hypothalamus and that soluble products of activated immunological cells induce a decrease in NA content in the hypothalamus.

Since hypothalamic catecholamine (CA) concentrations are rather stable under normal conditions (*4*), we studied turnover rates (*K*) and rate constants of amine loss (*k*) in control rats and in rats immunized with sheep red blood cells (SRBC) using α-methyl-*p*-L-tyrosine (α-MTP) (*5*). This drug blocks the rate-limiting step of CA synthesis, acting as a competitive inhibitor of tyrosine hydroxylase. At the time of the peak immune response to SRBC, that is, 4 days after antigenic challenge, the number of plaque-forming cells (PFC) was measured in the spleen, as was also the CA turnover rate in the hypothalamus. All data taken together indicated a tendency for a decreased NA turnover rate in immunized animals. Because our animals showed considerable variability in the magnitude of their response to SRBC, we considered the possibility that those animals that responded strongly to the antigen might show a more pronounced decrease in NA synthesis in the hypothalamus.

When the NA turnover rates in the hypothalamus of each animal were plotted against the corresponding number of PFC per spleen, it was apparent that the animals with a more evident decrease in NA synthesis in the hypothalamus were those that responded strongly to the antigen. The data were therefore assessed in terms of high and low responder animals. Those rats whose splenic PFC values were higher than the mean number of PFC were classified as high responders (high responders > mean PFC) and the others as low responders (low responders < mean PFC). By this criterion, high responders had two- to threefold more PFC in the spleen than low responders; about half of the number of animals fell in each category. This classification revealed that the high responders markedly decreased their NA turnover rates in the hypothalamus (Table 1) whereas the low responders had turnover rates almost equal to those of the nonimmunized controls.

No changes were noted on day 1 after immunization (data not shown), suggesting that this phenomenon was neither caused by stress during manipulation nor by the antigen itself. This view is reinforced by the fact that low responders

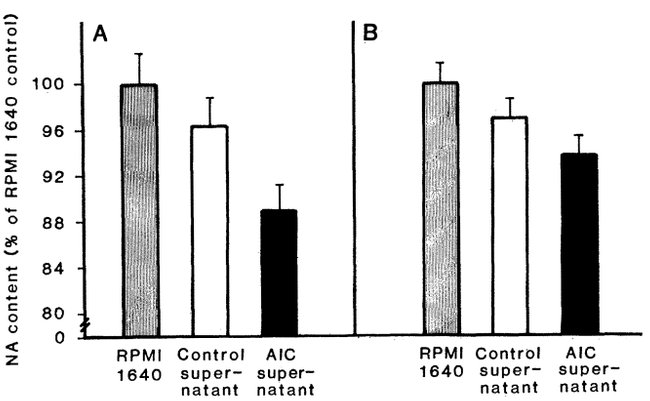

Fig. 1. Reduced NA content in (A) the hypothalamus and (B) the brainstem of rats after the injection of supernatants from activated immunological cells. One group of Holtzmann rats was injected intraperitoneally with 1 ml of the supernatant from activated immunological cells (*AIC*). These cells were obtained from the spleens of Lewis rats and were treated as described (*11*). Briefly, suspensions of 10^6 cells per milliliter in RPMI 1640 medium containing 10 percent heat-inactivated fetal calf serum were stimulated with Con A (5 μg/ml). After incubation of the mixture for 48 hours at 37°C, the supernatant was collected. Two other groups of rats were injected either with the same volume of RPMI 1640 medium or with control supernatant that was obtained as described above but Con A was added to the supernatant after collection. Two hours after the injection, the animals were killed and the hypothalamus, brainstem, and right hemisphere were removed as described in Table 1. Catecholamines were determined by high-performance liquid chromatography [column: 10 cm RP-18 Spheri 5 (Brownlee); mobile phase: 6.7 m*M* Na_2HPO_4; 13.3 m*M* citric acid; 2.5 m*M* octanesulfonic acid; 0.05 m*M* EDTA; 13 percent methanol, *p*H 3.3; flow rate: 1.3 ml/min] and electrochemical detection (detector potential against Ag/AgCl: 0.65 mV). Values are means ± standard error of the mean. Results are expressed as the percentages of those for rats injected with RPMI 1640 medium. The average NA values for this control ranged from 2913.78 ± 124.98 pg to 3506.70 ± 112.71 pg per milligram of tissue in the hypothalamus, and from 812.03 ± 13.94 pg to 960.73 ± 29.50 pg per milligram of tissue in the brainstem. In each experiment we used 13 to 16 animals per group. The NA content of the hypothalamus of rats injected with AIC supernatant differed significantly from controls injected with RPMI 1640 (Student's *t*-test, $P < 0.005$) or with control supernatant ($P < 0.05$). The NA content of the brainstem of rats injected with AIC supernatant differed significantly from the group injected with RPMI 1640 medium ($P < 0.02$).

received the same amount of antigen as the high responders. No changes in the NA turnover rate in the right hemisphere of the brain were detected during the immune response. Dopamine turnover was not affected, showing that the activity of NA neurons that project into the hypothalamus are selectively altered by the immunological events.

These results imply that signals released from activated immunological cells at the time of the peak of the immune response affected the activity of noradrenergic neurons. We searched for these signals by injecting into rats supernatants containing products released during the stimulation of immunological cells in vitro with concanavalin A (Con A). We expected that such supernatants, which are known to contain, among other products, monokines and several lymphokines, would acutely affect the NA in the hypothalamus. Two hours after administration of this material the NA of the hypothalamus decreased (see Fig. 1). The concentration of NA in the brainstem also decreased when compared with the control rats that received RPMI 1640 medium. No changes in NA were detected in the residual brain nor were changes in adrenaline and dopamine observed in any of the brain regions investigated.

In evaluating these results we considered the following. (i) Although CA concentrations in the CNS are known to be rather stable (*4*), products corresponding to only 10^6 or fewer activated immunological cells were potent enough to induce the decrease in the hypothalamic NA. (ii) The magnitude of NA decrease in the hypothalamus 2 hours after administration of the active supernatant corresponds to more than 50 percent of the decay in the hypothalamus NA content 2 hours after injection of α-MPT, which blocks NA synthesis (see Table 1). (iii) Apart from the results obtained with the appropriate controls, stress can be ruled out as a cause of NA decrease. During acute stress, epinephrine decreases markedly in the hypothalamus (*6*); however, epinephrine concentrations remained unchanged under our experimental conditions.

From these studies we conclude that the immune response can exert an inhibitory action on central noradrenergic neurons. This effect is probably produced by soluble mediators released by immunological cells because the administration of supernatants from cells stimulated in vitro can mimick within hours the events occurring 4 days after immunization in vivo as reflected by an acute reduction in basal concentrations of hypothalamic NA. These findings may enhance our understanding of neuroendocrine-immunoregulatory circuits. It is well known that NA has an inhibitory effect on several neuronal subsets in the hypothalamus including those of the medial part (*7*). It is therefore likely that decreased concentrations of NA in the hypothalamus are the cause of the increase in firing rates of neurons of the medial hypothalamus that occurs during the immune response (*2*).

A decrease in NA content was detected previously during the immune response in lymphoid organs, such as spleen (*3*). The present finding that central noradrenergic neurons also have a decreased monoamine content suggests that the same messengers mediate both phenomena and that they may be integrated in a sympathetic reflex mechanism during the immune response. The data showing that messengers from the immune system can alter brain activity could also be considered in a broader context. One of the major functions of the brain is to process information on changes in the external and internal environment detected by receptor organs. The present and previous evidence (*2*) shows that the brain is informed about intrusion of antigenic macromolecules or modified self-antigens. Immunological cells that express a huge repertoire of specific receptors for antigenic agents may, by way of releasing appropriate immunohormonal messengers, be the ultimate source of this information for the brain. The immune system may thus act as the peripheral receptor organ for this type of external or internal stimuli.

Table 1. Decreased rate of NA synthesis in the hypothalamus during the immune response. Female Holtzmann rats (2 to 3 months old) were divided into four groups. Two groups were injected intraperitoneally with 1 ml of saline and the other two with 1 ml of SRBC (5×10^9 cells). Four days later, one group injected with saline and one injected with SRBC received an intraperitoneal injection of α-methyl-*p*-L-tyrosine [200 mg/kg (*8*)]. The other two groups were injected with an equivalent volume of water to determine the initial levels of NA in control and immunized animals. Two hours later (*9*), animals were anesthetized with Nembutal (40 mg/kg) and the hypothalamus and right hemisphere were removed within 2 minutes after decapitation and frozen at −80°C. Spleens were used for determination of the immune response (PFC). The radiometric-enzymatic assay for measuring catecholamines as described by Da Prada and Zürcher (*10*) was applied to quantify NA. The method of Brodie *et al.* (*5*) was used for the calculation of turnover rates and constants. Values are means ± standard error. The NA turnover rates for high responders differ significantly from controls (Student's *t*-test, $P < 0.02$) and from low responders ($P < 0.05$).

Group	*N*	PFC per spleen	Initial NA concentration (ng/g)	*N*	PFC per spleen	NA concentration after α-MPT (ng/g)	Rate constant of amine loss (*k*) (per hour)	Turnover rate (*K*) (ng/g-hour)
All controls	19		2,910.27 ± 80.31	18		2,329.85 ± 90.52	0.1172 ± 0.0197	341.30 ± 57.20
All immunized	17	1,072,823 ± 198,000	2,918.10 ± 118.71	18	1,030,944 ± 189.000	2,424.54 ± 80.19	0.0895 ± 0.021	264.52 ± 59.33
Low responders	10	591,323 ± 117,800	3,076.43 ± 157.10	9	631,750 ± 203.000	2,366.00 ± 188.65	0.1307 ± 0.027	359.24 ± 89.68
High responders	7	1,765,578 ± 263,000	2,691.90 ± 154.55	9	1,470,764 ± 287.500	2,483.07 ± 151.31	0.0465 ± 0.021	125.25 ± 57.11

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Thrombin Stimulation of Guanosine 3′,5′-Monophosphate Formation in Murine Neuroblastoma Cells (Clone N1E-115)

Abstract. *Thrombin, the central regulatory enzyme in coagulation, when incubated in nanomolar concentrations with murine neuroblastoma cells produced a rapid and marked increase in tritiated guanosine 3′,5′-monophosphate (cyclic GMP) formation that was blocked by hirudin and competitively antagonized by dansylarginine* N-*(3-ethyl-1,5-pentanediyl)amide. Diisopropylphosphofluoridate-inactivated thrombin as well as the serine protease trypsin were markedly less potent and less effective than* α*-thrombin in producing this effect. Thrombin-stimulated cyclic GMP formation was inhibited by mepacrine and nordihydroguaiaretic acid but unaffected by indomethacin, suggesting that lipoxygenase metabolites of arachidonic acid are involved in the response. These results suggest that a thrombin-like protease in the brain may be involved with the function of neurons or that thrombin interactions with nerve cells, such as those following cerebral hemorrhage or other trauma of the central nervous system, may be important in the subsequent neuropathology.*

Clones of murine neuroblastoma cells in culture have been widely studied as model systems for neurobiology. Such clones possess many of the properties of normal differentiated neurons (*1*). Cells of one of these clones (N1E-115) respond to stimulation of the muscarinic or histamine H_1 receptor by increasing their concentration of guanosine 3′,5′-monophosphate (cyclic GMP). This response represents a convenient means by which receptor-mediated cellular activation can be investigated. During a study of the mechanism of receptor-mediated cyclic GMP formation, we found that the serine protease thrombin (E.C. 3.4.21.5), a pivotal enzyme in the regulation of hemostasis, also stimulated cyclic GMP formation in neuroblastoma cells. Here we characterize the nature of this effect and propose that thrombin receptors on neuroblastoma cells mediate this phenomenon. We also provide evidence that phospholipase A_2 activation and lipoxygenase metabolites are involved in this neuronal cyclic GMP response. The results suggest that thrombin plays a role in the neuropathology of cerebral hemorrhage or other traumas of the central nervous system in which the blood-brain barrier has been compromised.

Thrombin caused a rapid and marked increase in tritiated cyclic GMP formation by neuroblastoma cells with a peak around 30 to 60 seconds (Fig. 1). This time course is similar to that reported for the formation of cyclic GMP in this cell type in response to stimulation of the muscarinic and histamine H_1 receptors (*2*). Moreover, the response induced by thrombin was markedly dependent on extracellular Ca^{2+} as has also been observed for other agents (for example, carbachol, histamine, KCl, and Ca^{2+} ionophores) that enhance cyclic GMP

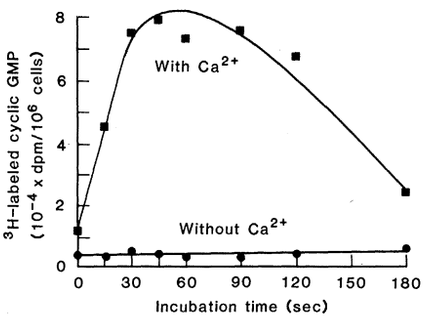

Fig. 1. Time course of the effect of thrombin (3 U/ml) on the formation of tritiated cyclic GMP in cultured murine neuroblastoma cells. Clone N1E-115 cells (subculture 9–11) were grown and assayed for relative changes in cyclic GMP formation with the use of a radioactively labeled precursor in intact cells as previously described (*18*). Symbols: ■, assay conducted in normal phosphate-buffered saline (PBS) solution containing 110 m*M* NaCl, 5.3 m*M* KCl, 1.8 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 2 m*M* Na_2HPO, 25 m*M* glucose, and 70 m*M* sucrose (*p*H 7.3 to 7.4, osmolality 335 to 340 mOsm); ●, assayed in PBS in the absence of Ca^{2+}, but containing 2 m*M* $MgCl_2$ and 1.0 m*M* EGTA with the other ions and conditions remaining unchanged. This figure depicts a representative result from one of four experiments.

synthesis (*2, 3*). The EC_{50} (median effective concentration) for thrombin ranged from 0.08 to 0.44 NIH units per mililiter (mean = 0.20 ± 0.04 U/ml in ten concentration-response studies with each point assayed in duplicate). Maximal thrombin concentrations (1 to 10 U/ml) increased cyclic GMP levels from 4.1 to 19 times more than the basal levels measured in these cells (mean = 11.5 ± 1.2 times; $N = 17$ experiments, each performed in duplicate or triplicate). Thrombin-stimulated increases in cyclic GMP were not affected by atropine ($10^{-7}M$) or pyrilamine ($10^{-6}M$), thus ruling out the possibility that muscarinic or histamine H_1 receptors were involved. In addition, a highly purified preparation of bovine thrombin (*4*) gave results indistinguishable from those obtained with thrombin from Sigma Chemical Company. Thus, the effect on cyclic GMP synthesis was not due to a contaminant of the commercially available preparation.

Thrombin consistently produced the most potent (EC_{50} = 1.0 to 1.5 n*M*) response whereas trypsin and diisopropylphosphofluoridate-inactivated thrombin (DIP-thrombin) were 50 to 100 times and 300 to 500 times less potent in stimulating this neuronal cyclic GMP response, respectively (Fig. 2A). Thus, this effect showed a marked degree of specificity for catalytically active thrombin; trypsin, another serine protease with a broad specificity, was much less potent and less effective in stimulating this response. That thrombin was the mediator of these effects was further established by the finding that hirudin, the specific thrombin inhibitor isolated from the leech salivary gland (*5*), inhibited thrombin-stimulated cyclic GMP formation in this preparation in a concentration-dependent fashion (Fig. 2B). In addition, the competitive inhibitor of thrombin dansylarginine *N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) shifted the response curve for thrombin concentration and cyclic GMP formation to the right in a parallel manner (Fig. 2C). Thus, DAPA was a competitive inhibitor of thrombin in this neuronal system and from a direct plot (*6*) of the dose ratio (DR) data for two concentrations of DAPA, we found a K_d (equilibrium dissociation constant) of $1.67 \times 10^{-8}M$ (standard error = $\pm 0.14 \times 10^{-8}M$). This value is in agreement with the competition of DAPA with thrombin in other systems (*7*). Finally, C-6 glioma cells that have β-adrenergic receptors that mediate the synthesis of both adenosine 3′,5′-monophosphate (cyclic AMP) and cyclic GMP (*8*) failed to produce cyclic GMP in response to thrombin (data not shown), suggesting