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Administration of mice of stressors, such as electric footshock or restraint results in decreases of spleen and thymus weights, and in a variable decrease in mitogen-stimulated lymphocyte proliferation. This variability can be decreased with careful attention of time factors when processing the tissues form large numbers of animal. However, a large part of it probably reflects the prior immunological history of the animals. When the stressors are applied acutely, the stress-induced deficits in lymphocyte proliferation are prevented by prior adrenalectomy, suggesting the involvement of adrenal glucocorticoids. The deficits observed after chronic stress, can be prevented by chronic treatment with thymosin fraction 5.

Administration to mice of supernatants from mitogen-stimulated rat spleen cells increases plasma corticosterone, but with little change in brain catecholamines. Parental administration of Newcastle disease virus (NDV) results in an activation of the pituitary-adrenal axis which requires an intact pituitary. Previous results that claimed these effects to be independent of the pituitary can be explained by incomplete

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hypophysectomy. The NDV administration also markedly depresses spleen cell proliferative responses. The increases in plasma corticosterone are parallelled by activation of certain brain neurotransmitter systems, notably the catecholamines. Concentrations of the norepinephrine metabolite MHPG and of tryptophan are increased in most brain regions, and dopamine and serotonin metabolites in a few. These changes resemble those observed with stressors such as footshock and restraint, suggesting that virus infection can be regarded as stressful. Moreover, the brain apparently monitors the process of immune activation, perhaps because it is sensitive to increases circulating concentrations of interleukin-1.

Final Report on Office of Naval Research Contract

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Our accomplishments on this contract have been in three major areas:

1. Pilot studies on stress-related changes in immune parameters.

2. Technical studies on the sources of variability in lymphocyte proliferation assays.

3. Changes in plasma corticosterone, and in cerebral catecholamine and indoleamine metabolism in response to Newcastle disease virus and cytokine administration.

Our progress in each of these areas is reviewed below.

1. Stress-related changes in immune responses

Considerble effort has been directed towards the task of establishing a consistently reproducible paradigm for the production of stress-related deficits in immune function. To this end, many experiments were performed using various stressors in the male CD-1 mouse. The stressors employed were electric footshock and restraint, two procedures used commonly in the field of stress research. These two procedures permit the degree of stress to be manipulated by, for example, altering the intensity of the footshock, or the duration of the period of stress. The emphasis was to determine the minimum stressful treatments that produce consistent results, both for humane reasons, and for reasons of experimental expedience. We typically use 20-22 footshocks (pulses of 1 second duration at 0.2 mA) in a 15 minute period or 40-45 in 30 minutes, or restraint for 30 to 120 minutes. We have also examined the relative effects of acute (one session) treatments, and chronic (repeated two to ten times) treatments.

Animals subjected to these treatments consistently show increased cerebral norepinephrine (NE) metabolism, as indicated by increased brain concentrations of 3-methoxy,4-hydroxyphenylethyleneglycol (MHPG), a major catabolite of NE, increased dopamine (DA) metabolism, as indicated by increased brain concentrations of dihydroxyphenylacetic acid (DOPAC), the major catabolite of DA, and increased metabolism of serotonin (5-HT), as indicated by increased concentrations of 5-hydroxyindoleacetic acid (5-HIAA), the major catabolite of 5-HT. Moreover, there are increases in whole brain tryptophan, and in plasma corticosterone.

The immunological analyses have indicated consistent reductions in spleen and thymus weights, particularly in the repeated or chronically stressed animals. Responsivity of splenic lymphocyte proliferation to mitogens, such as phytohemaglutinin (PHA), Concanavalin A (ConA), poke weed mitogen (PWM), and lipopolysaccharide (LPS) is reduced in most but not in all experiments. These results resemble those reported to the ONR by Maier. The variable response appears to arise at least in part from the considerable variability between animals encountered in these assays. We believe that this variability is most

likely due to incidental exposure of the animals to opportunistic infections and environmental antigens. Our results with NDV (see below) indicated that NDV administration severely suppressed responses to all mitogens.

In one series of experiments, we compared the effects of stressors on mitogen responses in adrenalectomized and sham-operated mice. Acute footshock treatment (30 minutes) significantly decreased [^{3}H]thymidine incorporation in response to Con-A and PHA (Figure 1). These effects was not observed in adrenealectomized mice. This result conflicts with the often cited study of Keller et al. (1983) who found significant stress-related deficits in adrenalectomized mice. However, the latter study was performed in rats, and used 20 hours of high intensity tail-shock. Thus there may be adrenal and extra-adrenal mediators of stress-related changes in lymphocyte proliferation. However, when we examined the responses in a chronic stress paradigm, we observed no effect on the lymphocyte proliferation in either intact or adrenalectomized mice.

We have also examined the ability of the administration of thymic extracts (thymosin fraction 5, TF5) to reverse the stress-related decreases in lymphocyte proliferation. In confirmation of the preliminary work of Hall et al., we have indeed observed such effects (Table 1).

2. Technical Studies on Lymphocyte Proliferative Responses.

In the course of these experiments, we experienced considerable variability in both cell viability and mitogen responsivity. Our experiments have established some reasons for this variability. When we conducted pilot experiments on the feasibility of freezing lymphocytes and shipping them to Washington for analysis, cell viability on thawing was very high, much higher than we had achieved in our stress experiments. The major reason for this was the delay involved in processing the tissue prior to freezing. When only a few animals are involved, this delay can be very short and cell viability is high. Our stress experiments must involve at least 16 animals for a minimum "n" of 8. and frequently we use considerably more animals than this. This increases the time delays in processing and the cell viability and mitogen responsivity suffers. We determined early on that it was important to disperse the spleen cells very soon after excision, rather than to save the cells and process them together which would be more convenient, and is acceptable for the neurochemical samples. Our most recent studies indicate that if the spleen cells are dispersed immediately after excision, and the cells are processed in small batches, excellent viability and mitogen responsivity is retained.

We had been in the habit of freezing our dispersed splenic lymphocytes (with a rate-freezing process) for shipment to Washington to perform the mitoegn assays. However, Dr. Adrian Gee of the University of Florida Department of Pediatric hematology, an expert in bone marrow transplants, advised us that lymphocytes populations were more stable than generally believed at room temperature, and that temperature changes of any kind might be more deleterious than the length of time outside of the body. This was consistent with our own observations with mouse spleen cells which suggested that cooling them to 0° resulted in deleterious effects. Therefore, we decided to take a radical approach and to ship the dispersed spleen cells unfrozen but stabilized against large changes in temperature. We did this by enclosing the cells in a standard

polystyrofoam container, and then inserting that container inside a second one, with cold-packs in the outer container. So that we could monitor the temperature changes encountered by the cells during shipment, we inluded a maximum-minimum thermometer in the inner package. Using Federal Express or similar couriers, the cells can be set up for incubation in Washington within 24 hours of removal from the mouse without being frozen. We have now conducted four experiments in this manner, and the results are dramatic. A controlled comparison in which cells from the same mice were shipped frozen and unfrozen separately clearly indicated the advantages of the non-freezing procedure. It is also far less labor-intensive, because the freezing and thawing of the cells takes considerable care, and time when large numbers of animals are involved. The major advantage is that the cell recovery and viability are is close to 100%. The results obtained with this procedure are similar to those obtained when the cells are cultured as soon as possible after harvesting.

3. Responses to Newcastle disease virus (NDV) administration.

We have adopted the model of Smith et al. (Science 218: 1311-12, 1982) using Newcastle disease virus (NDV) to investigate whether the CNS responds to the injection of virus, which might be regarded as a stressor. NDV is not infectious in the mouse (i.e. active viurs is not produced), but the RNA is replicated. The double-stranded replicative form acts as a potent stimulus for interferon and other cytokine production. We have been able to verify that there is a delayed increase in plasma concentrations of corticosterone with a peak at about 8 hours following injection as Smith et al. had reported. In eight separate experiments, we have not observed substantial increases in the plasma corticosterone of hypophysectomized mice following virus injection when the mice had been verified for the completeness of the hypophysectomy, by testing the plasma corticosterone response to restraint stress. This is a vital control procedure (omitted by Smith et al.) because it is well established that "hypophysectomized" rodents may retain some pituitary-adrenal function if any tissue is left in the sella tursica, even though this may not be detected by visual inspection (Moldow and Yalow, Proc. Natl Acad. Sci. 75: 994-998, 1979). While our initial results were in male mice of the CD-1 strain from Charles River, we have also replicated our results in female Swiss Webster mice from, Taconic Farms, the variety that Smith et al. used. Our experience with three separate batches of Taconic Farms mice is that the mice are poorly operated and we have been unable to obtain completely healthy animals from this supplier. This may have contributed to the results obtained by Smith et al. These results were published in a Technical Comment to Science (see publication list below).

Using NDV injection, we have, however, consistently observed alterations in the cerebral biogenic amine metabolism consistent with an activation of noradrenergic systems, and to a lesser extent dopaminergic and serotonergic systems. Brain tryptophan is also substantially increased (Figure 2). These responses did not differ substantially between hypophysectomized and intact mice, which rules out a role for ACTH or corticosterone. The results indicate that the brain does indeed respond acutely to an antigenic stimulus, so that it may be capable of coordinating an immune response. They also suggest that virus infection can indeed be regarded as stressful. The results were reported in full in Brain, Behavior, and Immunity (see publications list below).

We also observed that the mitogen responses of splenic lymphocytes of NDV-injected mice were severely depressed. This depression may indicate a source of variability in these assays, if exposure to any antigen or opportunistic infection also causes such a depression. This is consistent with our observation that the variability of this assay was markedly increased on two occasions when infections appeared in the colony.

In a separate series of experiments, we have investigated the ability of various thymic extracts to initiate cerebral biogenic amine responses. specifically to verify the report of Besedovsky et al. (1983) that injection of a concanavalin-A (Con-A)-stimulated spleen cell supernatant decreases the hypothalamic content of norepinephrine. This decrease of hypothalamic NE was interpreted to indicate an activation of noradrenergic systems in this region. These systems have been implicated in the control of the release of corticotropin-releasing factor (CRF), which in turn stimulates ACTH, and hence corticosterone release (i.e. the classic activation of the hypothalamic-pituitary-adrenal axis (Selye, 1950)). In three separate experiments, we have tested the effect of injections into mice of this same preparation or thymosin fraction V (Tf-5) on the cerebral content of biogenic amines and their catabolites. In no case have we observed consistent results supportive of Besedovsky's report. However, a much more sensitive index of noradrenergic activation is the production of the catabolite, MHPG. We have not found this compound to be altered significantly in any of our experiments, even though we have observed significant elevations of plasma corticosterone by both the lymphokine-containing preparation and Tf-5. These results were published in Brain, Behavior, and Immunity (see publications list below).

In one other related study we have investigated one potential mechanism by which Tf-5 might reverse stress-induced immunosuppression. Previous studies reviewed in the original application suggested that Tf5 can block the binding of dexamethasone to intact cells (lymphocytes). Thus, in collaboration with the laboratory of Dr. William Luttge at the University of Florida, which has extensive experience with glucocorticoid receptor assays, we investigated the ability of Tf-5 to compete for corticosterone binding to its receptors. Two experiments using partially purified cerebral glucocorticoid receptors showed absolutely no effect of Tf-5 on steroid binding. Thus the ability of Tf-5 to counteract glucocorticoid effects cannot be explained by a direct action on glucocorticoid receptors. This result suggests that if indeed Tf-5 is capable of interfering with steroid binding it may occur at the level of the membrane, possibly by blocking the passage of the steroid into the cell.

Publications

Dunn, A.J., Moreshead, W.V. & Powell, M.L. Responses of cerebral catecholamines and indoleamines to various stressors including injection with Newcastle disease virus. In: Synaptic Transmitters and Receptors, (Ed. Tucek, S., Barnard, E., Bartfai, T., Laduron, P., Lunt, G., Nahorski, S., Turner, A. & Wikler, H.), Prague: Academia (1987) pp.341-344.

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Dunn, A.J. Nervous system-immune system interactions: an overview. J. Receptor Res. 8: 589-607 (1988).

Presentations

Nervous system-immune system interactions: an overview. Presented at the Third Swiss Workshop of Methdology in Receptor Research, Grindelwald, Switzerland, March 22-26, 1987.

Psychoneuroimmunology for the psychoneuroendocrinologist. Presented at the Annual Meeting of the International Society for Psychoneuroendocrinology, Chapel Hill, N.C., July 2, 1987.

A virus-activated stress response in mice: cerebral biogenic amines, plasma corticosterone, and lymphocyte proliferation. Poster presentation at the Society for Neuroscience Annual Meeting, New Orleans, November 20, 1987. (see abstract above).

Nervous system-immune system interactions: an overview. Presentation at the Society for Neuroscience Psychoneuroimmunology Dinner, New Orleans, November 20, 1987.

Graduate Students

Bryan L. Spangelo was supported on the Subcontract to George Washington University for 2-3 months to perform some of the immunological assays.

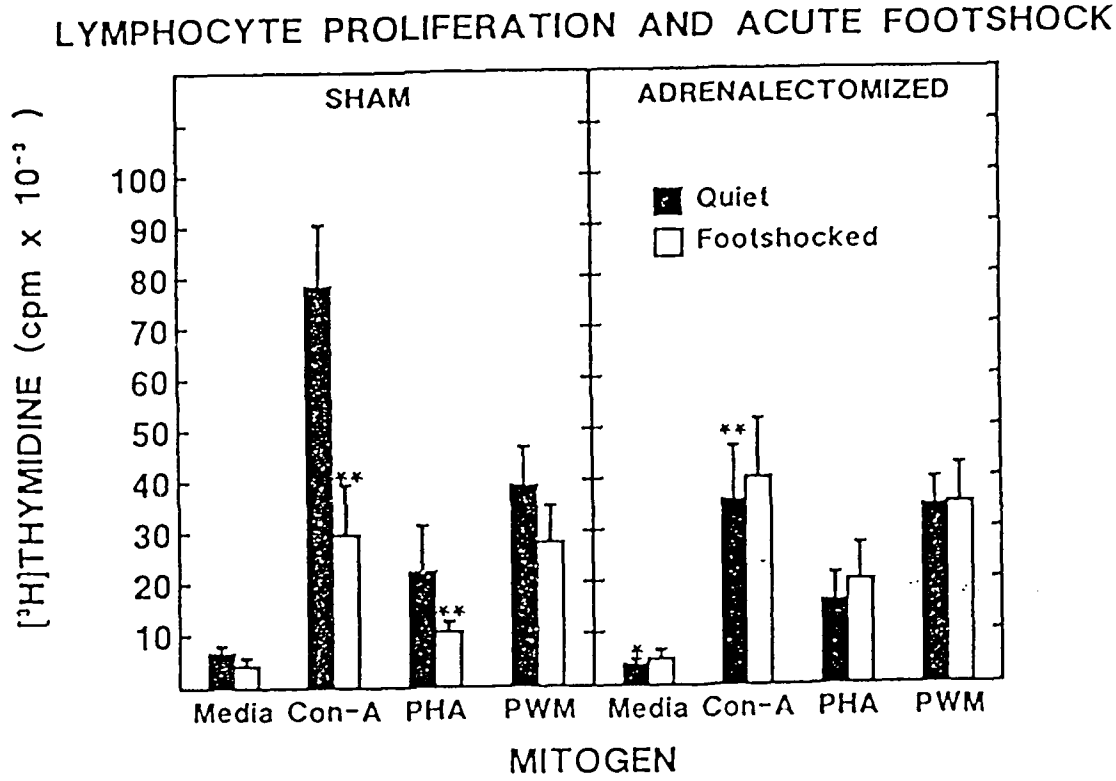

Figure 1 Proliferative responses in mice subjected to electric footshock. Adrenalectomized or sham-operated mice were subjected to unsignalled inescapable footshock (40 shocks 0.25 mA, 1 sec duration) in 30 minutes. The responses of isolated spleen cells to Con-A and PHA *in vitro* were determined as indicated in Dunn et al. (1987b). **Different from quiet control mice ($P < 0.01$). *Different from sham-operated mice ($P < 0.05$).

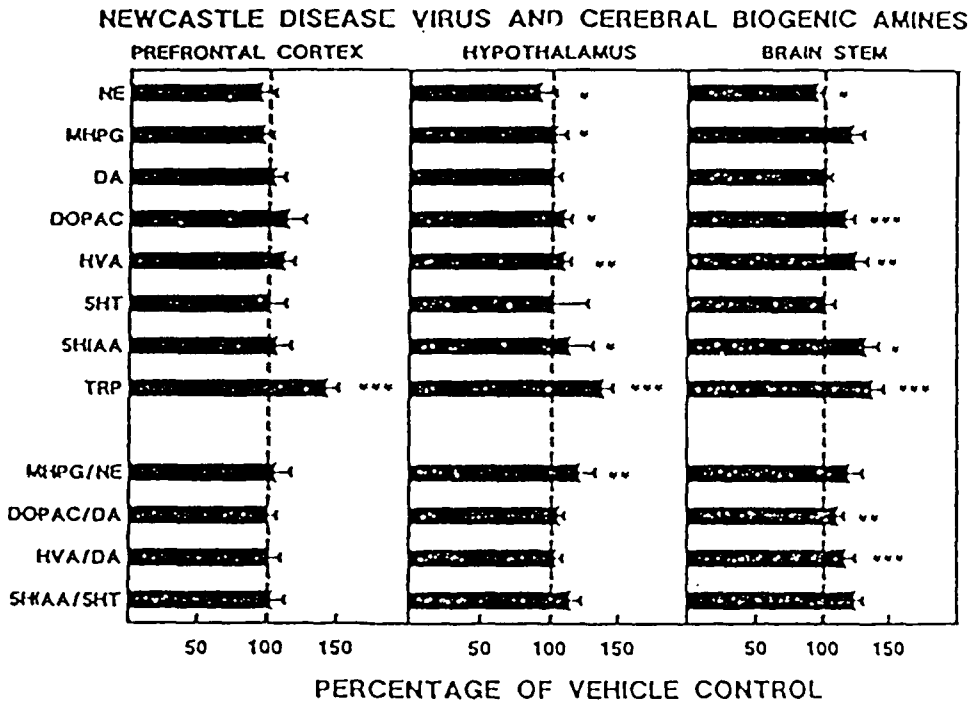

Figure 2 The effect of Newcastle disease virus (NDV) on concentrations of brain biogenic amines and catabolites. NDV was injected into mice ip 8 hours prior to decapitation. NE - norepinephrine, MHPG - 3-methoxy,4-hydroxyphenylethyleneglycol, DA - dopamine, DOPAC - 3,4-dihydroxyphenylacetic acid, HVA- homovanillic acid, 5-HT - 5-hydroxytryptamine, 5-HIAA - 5-hydroxyindoleacetic acid, Trp - tryptophan. Amines and catabolites were determined by HPLC with electrochemical detection. *Significantly different from vehicle control (*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$). Data from Dunn et al. (1987b).

TABLE I

Thymosin Fraction 5 Effects on Restraint-Induced Mitogenicity

	[^{3}H]Thymidine cpm						
	Media	Con-A	SI	PHA	SI	PWM	SI
Quiet	635 ±50	12603 ±3923	19.0 ±5.1	1723 ±228	2.8 ±0.5	3658 ±812	5.3 ±1.7
Restrained	659 ±251	7688 ±4430	6.5* ±2.1	1581 ±774	1.8* ±0.3	3203 ±1751	31.0 ±0.7
Restrained + TF-5	645 ±243	11210 ±5914	12.4+ ±3.0	2378 ±1072	3.5+ ±0.6	4259 ±2006	5.0 ±1.3

Mice were restrained for 1 hour twice daily for 8 days. They were injected with thymosin fraction five (TF-5: 0.5 mg/mouse IP) or saline before each period of restraint. SI - stimulation index (i.e. Con-A/media, etc.). *Significantly different from quiet ($p < 0.05$), +significantly different from restrained ($p < 0.05$).

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