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Hypothalamic–immune interactions

II. THE EFFECT OF HYPOTHALAMIC LESIONS ON THE ABILITY OF ADHERENT SPLEEN CELLS TO LIMIT LYMPHOCYTE BLASTOGENESIS

T. L. ROSZMAN, R. J. CROSS, W. H. BROOKS* & W. R. MARKESBERY† *Division of Experimental Pathology, Department of Pathology, *Division of Neurosurgery, Department of Surgery and †Department of Pathology and Neurology and the Sanders-Brown Research Center on Aging, University of Kentucky Medical Center, Lexington, Kentucky, U.S.A.*

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Summary. Animals with electrolytic pre-optic and anterior hypothalamic (AHT) lesions show impaired mitogen-induced lymphocyte blastogenesis which is restored by removal of a population of spleen cells with macrophage-like properties. Although suppressor macrophages are detectable in normal and control rats, substantially more activity is present following AHT destruction. Abrogation of lymphocyte activation does not result from increased numbers of splenic macrophages. These data indicate that one mechanism by which neuroimmunomodulation occurs is by induction of a qualitative alteration in the function of naturally occurring suppressor macrophages.

INTRODUCTION

Normal immunological function results from specific and/or non-specific counterbalancing regulatory mechanisms, e.g. genetic (Benacerraf & McDevitt, 1972; McDevitt, 1980) and helper-suppressor cell controls (Gershon, 1974). In addition to these 'internal' regulators, it is now evident that other factors, existing beyond the usual confines of the immune system, also are capable of modulating immune responsiveness (Cross, Markesbery, Brooks & Roszman, 1980a, 1980b). Recently, we have confirmed and extended earlier reports (Isakovic & Jankovic, 1973; Stein, Schiavi & Camerino, 1976; Keller, Stein & Camerino, 1980) demonstrating that the central nervous system (CNS) is among these 'external' influences. Destructive lesions of the pre-optic and anterior hypothalamic area (AHT) result in markedly diminished *in vitro* cell-mediated immune responsiveness and thymic involution which is unrelated to corticosteroid production and release (Cross *et al.*, 1980a). Electrolytic lesions in the amygdala and hippocampus enhance reactivity (Cross *et al.*, 1980b). These findings, together with those demonstrating immunosuppression associated with stress (Solomon, 1969; Spry, 1972), bereavement (Bartrop, Lazarus, Luckhurst, Kiloh & Penny, 1977), and CNS disorders (Hoffman, Robbins, Nolte, Gibbs & Gajdusek, 1978; Morrel, 1979), suggest the presence of a 'neuroimmunomodulatory system' which is capable of influencing normal host immunocompetence.

Correspondence: Dr T. L. Roszman, Division of Experimental Pathology, Department of Pathology, University of Kentucky Medical Center, Lexington, Kentucky 40536, U.S.A.

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The purpose of the present report is to extend this concept by investigating the influence of the CNS on endogenous suppressor cell function. These results demonstrate that diminished lymphocyte reactivity following induction of lesions in the anterior hypothalamus results from qualitative changes in naturally occurring suppressor macrophages.

MATERIALS AND METHODS

Animals

Male Fischer 344 rats, age 65–90 days (Harlan Industries, Indianapolis, Ind.) were used in these experiments.

Induction of brain lesions

Details of the surgical techniques and stereotaxic co-ordinates used for cerebral lesioning have been presented elsewhere (Cross *et al.*, 1980a). Briefly, rats were anaesthetized with chloral hydrate (35 mg/kg) and placed in a Kopf stereotaxic apparatus. Using co-ordinates of Pellegrino, Pellegrino & Cushman (1979), bilateral electrolytic lesions were placed in the AHT. Control lesions were placed in the frontal grey matter. Brains were removed and lesion sites determined by microscopic study. Experimental animals whose lesions did not fall within the pre-optic and anterior hypothalamic areas were excluded. No alterations in normal behaviour pattern, general health, body weight or food and water intake were observed among the groups of animals. The mortality rate was $< 10\%$ and was confined to the immediate post-operative period.

Preparation of spleen cell suspensions

Animals were anaesthetized with methyoxyflurane (Pitman-Moore Inc., Washington Crossing, N.J.) and immediately killed by cervical dislocation. Spleens were aseptically removed and dispersed cell suspensions prepared as previously described (Cross *et al.*, 1980a). One aliquot of these suspensions was passed over a glass bead column and non-adherent and adherent fractions collected (Shortman, Williams, Jackson, Russell, Byrt & Diener, 1971). The recovery of cells from the column was $79 \pm 5\%$. The percentage of thymus-derived lymphocytes (T cells), bone-marrow-derived lymphocytes (B cells) and macrophages present in the non-fractionated and glass bead column fractionated spleen cells was determined as previously described (Roszman, Brooks, Markesbery, Aziz & Bigner, 1981). An indirect fluorescent antibody assay was employed to quantify T cells using a horse anti-rat thymocyte serum extensively absorbed with aggregated rat serum, kidney, liver, bone marrow and red blood cells according to the method of Viet & Feldman (1976). B cells, were quantified employing fluroescein conjugated $(Fab')_2$ fragments of a rabbit anti-rat immunoglobulin serum (Cappel Laboratories, Downington, Pa.). The percentage of macrophages in the cell suspensions was determined by the non-specific esterase stain (Yam, Li & Crosby, 1971).

Spleen cell cultures

Spleen cell cultures were prepared as previously described (Cross *et al.*, 1980a). Briefly, 250 μl of the spleen cell suspensions were pipetted into microtitre plates (Falcon number 3040, Oxnard, Calif.) and stimulated with concanavalin A (Con A, Sigma Chemical Co., St Louis, Mo.). Cell cultures were performed in triplicate. The culture plates were incubated at 37° in a 5% CO_2-air atmosphere for 56 hr at which time 0·25 μCi [^{3}H]-thymidine (New England Nuclear, Boston, Mass.; specific activity 6·7 Ci/mmol) was added in a volume of 10 μl. The cells were harvested 16–18 hr later using an automatic cell harvester (Microbiological Associates, Bethesda, Md.) and the [^{3}H]-thymidine incorporation into DNA was assayed by liquid scintillation counting. The data were analysed by the two-tailed Student's t test for independent means.

In some experiments varying numbers of glass bead adherent cells were added to non-adherent cells and stimulated with Con A. In addition, the adherent cells were either treated with absorbed horse anti-rat thymocyte serum or irradiated before addition to the non-adherent cells. The 5×10^6 adherent cells were incubated for 1 hr at 37° in 0·5 ml of antiserum or absorbed normal horse serum in the presence of 0·1 ml of a 1/5 dilution of guinea-pig complement (Grand Island Biological Co., Grand Island, N.Y.). The cells were washed twice in RPMI 1640, counted, viability determined by trypan blue dye exclusion and resuspended to a concentration of 5×10^6/ml. For irradiation, adherent cells at a concentration of 1×10^7/ml in RPMI 1640 without bicarbonate were placed in 10×35 mm plastic dishes (Lux Scientific Corporation, Thousand Oaks, Calif.) and exposed to a cobalt-60 source at an output dose rate of between 125 and 150 rad/min using a Picker C-9 unit. The source surface distance was 80, the field size 35 cm $\times$ 35 cm and the energy 1·25 MeV. After irradiation the cells were

washed twice and resuspended to a concentration of 5×10^6/ml.

RESULTS

The results presented in Table 1 indicate that the non-fractionated spleen cell blastogenic response of animals with AHT lesions is diminished when compared with that of control and normal animals. Importantly, the spleen cell responsiveness to Con A of control animals did not differ from that of normal animals. To investigate if this observed decrease in blastogenic reactivity of spleen cells from AHT lesioned animals could be alleviated, these cells were passed through a glass bead column to remove adherent cells. Removal of an adherent subpopulation of spleen cells resulted in increased Con A-induced responsiveness for all groups. Significantly improved reactivity, however, was observed only in AHT-lesioned animals. These results suggest either the presence of an adherent suppressor cell in the spleen cell population or a marked enrichment of T cells in the glass bead non-adherent cell fraction. To distinguish between these two possibilities the following experiments were performed. The percentage of T and B cells were determined in the various spleen cell populations and fractions. The results indicated that the percentage of T cells ranged from 60% to 65% and the B cells from 25% to 29% in the non-fractionated and non-adherent fraction cells from either AHT or control animals. Thus, the increased mitogenic activity exhibited by the non-adherent cells is not because of an increase in the number of Con A-responsive cells. Cell mixing experiments were performed to determine if the adherent cells had suppressor activity (Table 2). Addition of various numbers of adherent cells obtained from glass bead columns to autologous non-adherent cell fractions resulted in a dose-dependent decrease in blastogenic activity indicative of the presence of suppressor cells. Similar results were obtained when the adherent cells from either animals

Table 1. Con A responsiveness of non-fractionated and glass-bead non-adherent spleen cells from rats with AHT lesions

	Spleen cells	c.p.m./Well
AHT lesions	Non-fractionated	14,264* ±3322
	Non-adherent	40,050 ±4186
Control lesions	Non-fractionated	36,571 ±4305
	Non-adherent	42,812 ±4245
Normal	Non-fractionated	39,905 ±4402
	Non-adherent	44,956 ±9729

* Results represent the mean ± SEM of ten separate experiments using 1×10^6 cells/well. The response of AHT lesioned non-fractionated spleen cells was significantly different ($P < 0.02$) from the response of AHT lesioned non-adherent spleen cells. The responses of spleen cells (non-fractionated or non-adherent) from control lesioned or normal animals did not differ significantly ($P > 0.05$). Comparison of the response of AHT lesioned non-adherent spleen cells to the response of non-adherent spleen cells from control lesioned or normal animals revealed no difference ($P > 0.05$).

Table 2. The effect of glass bead adherent cells from normal and AHT lesioned animals on the response of their respective non-adherent cells to Con A (5 μg)

	Normal		AHT lesioned	
Cell source	c.p.m.	I (%)*	c.p.m.	I (%)
1×10^6 Non-adherent	28,569†		22,668	
$1{\cdot}5 \times 10^6$ Non-adherent	25,799		23,521	
1×10^6 Non-adherent + 1×10^5 adherent	28,922	0	14,028	38
1×10^6 Non-adherent + 2×10^5 adherent	24,367	15	9436	58
1×10^6 Non-adherent + 5×10^5 adherent	15,002	47	4644	80

* Percentage inhibition (I) = 100 × [(Cultures, c.p.m., with non-adherent + adherent cells)/Non-adherent cells, c.p.m.)] − 100.

† SEM did not deviate by > 10%.

Table 3. The effect of irradiation and anti-thymocyte serum on the suppressor activity of adherent cells from AHT-lesioned animals*

Cell treatment	Inhibition (%)‡
1×10^6 Non-adherent† + 5×10^5 adherent	$58 \pm 4{\cdot}2$
1×10^6 Non-adherent + 5×10^5 irradiated (2500 rad) adherent	$59 \pm 3{\cdot}5$
1×10^6 Non-adherent + 5×10^5 antiserum and C-treated adherent	$67 \pm 4{\cdot}1$
1×10^6 Non-adherent + 5×10^5 C only treated adherent	$69 \pm 13{\cdot}0$

* Results represent the mean ± SEM of three separate experiments.
† Non-adherent cells were stimulated with 5 μg Con A.
‡ Percentage inhibition = 100 × [(Cultures, c.p.m., with non-adherent plus adherent cells)/(cultures, c.p.m., with non-adherent cells alone)] − 100.

with AHT lesions or normal animals were added to the opposite non-adherent cell fractions (data not shown).

Further experiments were performed to characterize the suppressor cell present in the adherent cell fraction from the glass bead columns (Table 3). Although these studies utilized spleen cells obtained from animals with AHT lesions identical results were obtained with normal animals. Treatment of the adherent cells with anti-rat thymocyte serum or 2500 rad failed to reduce their suppressor effect (Table 3). These data indicate the presence of a suppressor cell with macrophage-like characteristics in the adherent cell fraction. These results are consistent with the observations of others that naturally occurring suppressor macrophages in the rat are responsible for inhibition of lymphocyte reactivity to mitogens and alloantigens (Bash & Waksman, 1975; Mattingly, Eardley, Kemp & Gershon, 1979).

The percentage of macrophages as determined by non-specific esterase staining in non-fractionated spleen cell suspensions from control animals was $6{\cdot}5\% \pm 0{\cdot}7\%$ and from animals with AHT lesions was $5{\cdot}4\% \pm 0{\cdot}9\%$. Although AHT lesions did not increase the percentage of splenic macrophages, results in Fig. 1 illustrate that on a per cell basis macrophages from animals with AHT lesions were significantly more suppressive than those from control animals. These data suggest that AHT lesions induce qualitative changes in the ability of endogenous macrophages to suppress lymphocyte function.

DISCUSSION

Immunological studies have shown that macrophage-like suppressor cells are found in the spleens of rats which abrogate lymphocyte function (Bash & Waksman, 1975; Mattingly *et al.*, 1979; Rode, Votila & Gordon, 1978). Moreover, the suppressive activity of this cell is significantly increased in animals harbouring experimentally-induced malignant tumours or suffering from viral and/or bacterial infection (Glaser, Kirchner & Herberman, 1975; Kryisbeek, Zijlstra & Zurcher, 1978; Camus, Dessaint, Fischer & Caperon, 1979; Mattingly *et al.*, 1979). Increased activity is related to significant increases in the number of splenic macrophages (Webb, Brooks & Baldwin, 1980). The present study demonstrates that the effect of AHT lesions on lymphocyte activity also is mediated by naturally occurring splenic suppressor macrophages. It differs, however, in that quantitative changes in splenic macrophages were not observed. Thus, the magnitude of lymphocyte responsiveness is influenced by neurally induced qualitative changes in suppressor macrophage function unrelated to the number of cells.

Of equal importance, the data obtained in this study lend additional credence to the concept of 'neuroimmunomodulation'. Two hypotheses may be offered to explain the consequences of this neuroimmunomodulatory influence on immune regulation. The first suggests that the CNS regulates immune responsiveness independently of and before any immunological activity. Thus, the CNS influences suppressor cell function before antigenic stimulation thereby altering the potential of immunological responsiveness. Whether this influence of macrophage function is mediated by alterations in the concentrations of circulating non-corticoid hormones (Pierpaoli, Baroni, Fabris & Sorkin, 1969) and/or the innervation of the immune system (Bulloch & Moore, 1980; Giron, Crutcher & Davis, 1980) remains to be established. Nevertheless, such interrelations would explain the

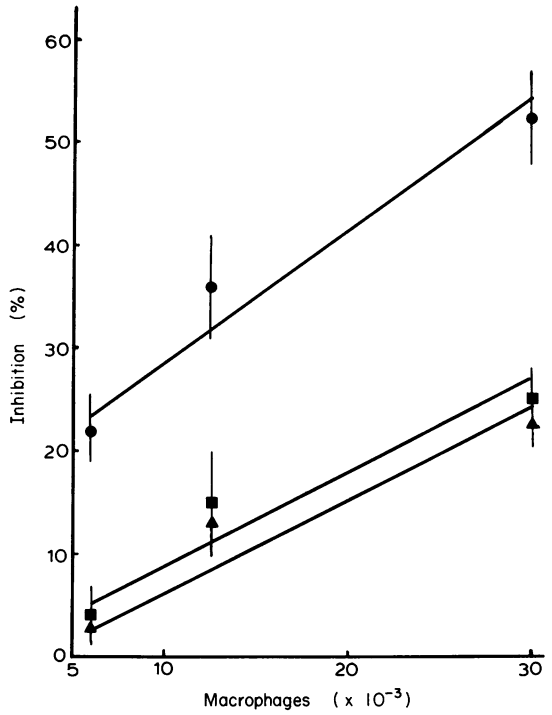

Figure 1. Effect of macrophages from normal (■), AHT-lesioned (●), and control-lesioned (▲) animals on the Con A responsiveness of non-adherent cells. Adherent cells were obtained by elution from glass-bead columns with 0·02% EDTA as previously described and the number of macrophages quantified by the non-specific esterase stain (Yam, Li & Crosby, 1971). Various numbers of macrophages were added to 1×10^6 of their respective non-adherent cells. These cells were stimulated with 5 μg of Con A to undergo blast transformation. The results represent the mean ± SEM of three separate experiments. The values obtained with macrophages from animals with AHT lesions differed from those from control lesioned or normal animals ($P < 0{\cdot}05$). Percentage inhibition = 100 × [(culture, c.p.m., with non-adherent cell plus macrophages)/(cultures, c.p.m., with non-adherent cells alone)] − 100.

immunosuppression observed during emotional stress and bereavement and explain fluctuations in *in vivo* and *in vitro* immunological probes routinely reported in normal individuals sequentially tested at daily and/or weekly intervals (Roberts, 1980).

Whereas the above hypothesis implies a considerable, if not absolute, degree of autonomy of the CNS in regards to regulation of immune responsiveness, the second hypothesis suggests the presence of a balanced neuroimmunological network. Accordingly, as the immune response is triggered, activity within the hypothalamus increases. This in turn alters the concentrations of circulating hormones and/or autonomic nervous system (Besedovsky, DelRay & Sorkin, 1979) activity which are capable of influencing immunocompetence. Indeed, support for this hypothesis is gained from the observation that neural activity within the hypothalamus is increased during the peak of immune responsiveness (Besedovsky, Sorkin, Felix & Haas, 1977). Therefore, an afferent/efferent neuroimmunological 'pathway' is hypothesized which allows the CNS to regulate immunological responsiveness by influencing suppressor macrophage function subsequent to antigenic challenge.

Although these hypotheses and relationships among the CNS, endocrine, and autonomic nervous system remain to be proven, the finding that discrete lesions with the CNS result in marked modulation of immune function by qualitatively altering naturally occurring suppressor macrophage function indicates the existence of a delicately balanced neural-immune system. Continued exploration of these facets of neuroimmunomodulation offers an excellent opportunity to analyse the intricacy of this system.

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