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Hypothalamic–immune interactions: neuromodulation of natural killer activity by lesioning of the anterior hypothalamus

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Summary. Placement of bilateral electrolytic lesions in the preoptic-anterior hypothalamic area (AHT) of Fischer 344 rats results in decreased splenic NK activity as compared to control and normal animals. Animals with AHT lesions have a decrease in NK activity 4 and 7 days after lesion placement, with a return to normal activity by day 14. Fractionation of spleen cells on glass bead columns results in non-adherent and adherent cell fractions with NK activity. AHT lesions affect NK activity only in the adherent cell fraction. The removal of macrophages from this cell fraction did not restore NK activity. Moreover, this NK activity is not the result of cytotoxic macrophages. Hypophysectomy decreases NK activity in lesioned and non-lesioned animals, suggesting the influence of pituitary factors. These data indicate that the anterior hypothalamus is capable of modulating the cytotoxic activity of NK cells. Thus, neuroimmunomodulation may be a potential factor in susceptibility to some disease states such as viral infections and neoplasia.

INTRODUCTION

Recently it has been shown that regulation of the immune response is not limited to factors or networks endogenous to the immune system (Benacerraf & McDevitt, 1972; McDevitt, 1980; Gershon, 1974; Jerne, 1974), but also includes mechanisms that emanate from the central nervous system (CNS) (Besedovsky & Sorkin, 1977). These effectors include hormones (Dougherty *et al.*, 1964; Vischer, 1972; Ambrose, 1970; Filipp & Mess, 1969; Fabris, 1973; Fabris, Pierpaoli & Sorkin, 1971; Pierpaoli & Maestroni, 1977, 1978), neurotransmitters (Devoino *et al.*, 1975; Bliznakov, 1980), endorphins (Gilman *et al.*, 1982; Johnson *et al.*, 1982) or sympathetic nerve pathways (Besedovsky *et al.*, 1979; Kasahara *et al.*, 1977; Williams *et al.*, 1981; Williams & Felten, 1981; Giron, Crutcher & Davis, 1980). Additional support is found for a concept of neural–immune interactions in reports which indicate that perturbations of the CNS by electrolytic lesions in the hypothalamus and the limbic system induce a variety of immune alterations. These include: (i) changes in lymphoid tissue architecture (Isakovic & Jankovic, 1973; Cross *et al.*, 1980; Brooks *et al.*, 1982), (ii) decreased susceptibility to induction of delayed-type hypersensitivity (Jankovic & Isakovic, 1973), (iii) decreased anaphylactic reactions (Luparello, Stein & Park, 1964), (iv) impaired or enhanced non-specific lymphocyte activation (Cross *et al.*, 1980; Brooks *et al.*, 1982; Keller *et al.*, 1980; Cross *et al.*, 1982), (v) decreased responsiveness to transplantation antigens (Dann, Wachtel & Rubin, 1979), and (vi) decreased humoral response to protein antigens (Jankovic & Isakovic, 1973; Tyrey & Nalbandov, 1972).

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Several clinical correlates for neuroimmunomodulation also exist. For example, depressed immune responsiveness in patients with cerebral tumours (Brooks *et al.*, 1972), Huntington's disease (Morrell, 1979) or Guamanian-Parkinsonism-dementia complex (Hoffman *et al.*, 1978) indicate that diseases of the CNS are associated with changes in immune competence. Additionally, individuals with psychosis (Kovaleva, Bonartsev & Prilipko, 1977), bereavement and depression (Bartrop, Lazarus & Luckhurst, 1977), and emotional stress also exhibit impaired lymphocyte reactivity and antibody production (Solomon, 1969a). Investigations of experimental animals stressed by overcrowding or avoidance conditioning display impaired immune responsiveness (Rassmussen, Spencer & Marsh, 1959; Solomon, 1969b), decreased resistance to viral infections (Rassmussen, 1969), and an increased incidence of neoplasia (Riley, Fitzmaurice & Spackman, 1981), further suggesting a link between behaviour and disease susceptibility.

Numerous reports suggest that immune surveillance for viral infections and neoplastic disease is provided by either cytotoxic T lymphocytes (Martz, 1974) or natural killer (NK) cells (Herberman & Ortaldo, 1981; Herberman & Holden, 1978). NK cells are cytotoxic for a variety of cells including virally transformed (Williams, Leifer & Moore, 1977), lymphoma (Nunn *et al.*, 1976), and those derived from solid tumours (Brooks *et al.*, 1980).

The purpose of this report is to determine if lesions in the preoptic-anterior hypothalamic area (AHT) modulate NK activity.

MATERIALS AND METHODS

Animals

Hypophysectomized and non-hypophysectomized male Fischer 344 rats (Charles River, Wilmington, MA), age 65–90 days, were used in these experiments. Hypophysectomy (HX) was performed 3–4 weeks prior to use and HX rats were maintained on 5% glucose solution.

Induction of brain lesions

Bilateral, electrolytic lesions were placed in the AHT as reported previously (Cross *et al.*, 1980). Rats with lesions in the frontal grey matter (FC) served as controls. Rats were anaesthetized with chloral hydrate (35 mg/kg). Lesions were placed using direct current (1·5 mA for 15 sec) through a noble-platinum electrode at the following coordinants: AHT—1·2 mm anterior to the bregma, 0·7 mm lateral of the midline and 7·4 mm below the dura; FC—4·0 mm anterior to the bregma, 3·0 mm lateral of the midline and 1·5 mm below the dura.

At the time of death, the brain was removed, fixed in 4% formalin, and sectioned in a coronal plane. Haematoxylin and eosin-stained sections were examined for location and extent of the lesions. All animals whose lesions did not fall within the AHT as previously defined (Cross *et al.*, 1980) were discarded. Mortality was $< 10\%$ and confined to the first 24 hr following surgery.

Preparation of spleen cell suspensions

Animals were anaesthetized with methoxyflurane (Pitman-Moore Inc., Washington Crossing, NJ) and killed by cervical dislocation. Spleens were removed and teased into dispersed cell suspensions in RPMI-1640 (Gibco, Grand Island, NY). An aliquot was removed and set aside on ice for later evaluation of NK activity. The remainder of the cells were fractioned over a glass bead column, and non-adherent (GBNA) and adherent (GBA) cell fractions were collected (Shortman *et al.*, 1971). Approximately 58% of the cells were recovered in the GBNA cell fraction and 12% were recovered in the GBA cell fraction. An aliquot of adherent cells was incubated with 200 mg of carbonyl iron (Pfaltz and Bauer, Sanford, CT) in 10 ml of RPMI-1640 supplemented with 5% foetal calf serum (FCS; Gibco, Grand Island, NY) for 1 hr at 37°. A magnet was applied to the bottom of the flask and the suspended cells were transferred to plastic 50 ml conical tubes. The iron filings remaining in the flask were resuspended in 10 ml of RPMI-1640 with 5% FCS, the magnet applied, and the cells transferred to a second 50 ml conical tube. The cells were centrifuged, washed, and resuspended in 5 ml of medium. Any remaining iron filings were removed from this cell suspension by employing the magnet, the cells transferred to another tube, washed once and counted. The carbonyl iron-treated cell fraction contained $< 1\%$ macrophages as determined by non-specific esterase staining (Yam, Li & Crosby, 1971).

Natural killer cell assay

NK activity was quantified using a ^{51}Cr-release assay. Spleen cells were suspended in RPMI-1640 supplemented with penicillin, streptomycin, vitamins, glutamine, non-essential amino acids, 10% heat-inactivated

FCS and adjusted to 1×10^7 cells/ml. All cell populations were incubated for 2 hr at 37° prior to assay.

Adenovirus type 2 transformed rat fibroblasts (F4) which served as NK target cells (obtained from Dr J. Sheil, University of Kentucky) were labelled with 250 μCi sodium ^{51}chromate for 1 hr at 37°. Target cells were washed three times, counted, and adjusted to 1×10^5 cells/ml. NK activity was measured by adding 100 μl of F4 target cells to a 96-well round-bottom microtitre plate. Spleen cells and target cells were added at an effector: target ratio of 100:1, 50:1, 25:1, and 12·5:1. Supernatant fluids were harvested after 18 hr using the Titertek supernatant collection system (Flow Laboratories, McLean, VA) and released ^{51}Cr was counted in a Beckman Gamma 8000. Percent cytotoxicity was calculated using the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{c.p.m. experimental release} - \text{c.p.m. spontaneous release}}{\text{c.p.m. maximum release} - \text{c.p.m. spontaneous release}} \times 100$$

Lytic units were calculated by fitting regression lines using percent cytotoxicity obtained at each effector: target cell ratio. One lytic unit was arbitrarily set at 20% cytotoxicity and the number of effector cells required to obtain this level of killing was obtained from the regression line. Lytic units/10^6 cells were calculated accordingly. Regression lines were generated for all four effector cell populations (unfractionated, GBNA, GBA, and carbonyl iron-treated GBA) from each of the three groups of animals (AHT, FC, and normal). The data were analysed by the two-tailed Student's *t*-test for independent means.

RESULTS

Effects of AHT lesions on NK activity

Experiments were performed to determine the effects of AHT lesions on NK activity. Four days after placement of AHT lesions splenic NK activity was determined. As shown in Fig. 1, rats with AHT lesions display impaired NK activity as compared to FC-lesioned and normal animals. This diminished response persisted through a range of effector: target cell ratios. FC-lesioned animals did not differ from normals in their NK activity.

Kinetics of AHT lesion-induced NK impairment

The kinetics of diminished NK activity were determined in animals killed 4, 7 and 14 days after lesioning.

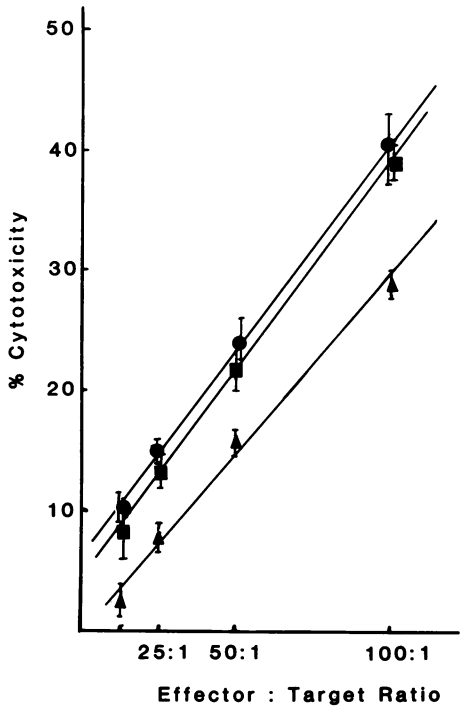

Figure 1. Splenic NK activity in AHT (▲), FC (■) and normal (●) animals at increasing effector: target cell ratios 4 days after lesion placement. NK activity in AHT-lesioned animals is significantly different from FC-lesioned ($P < 0{\cdot}01$) and normal animals ($P < 0{\cdot}001$). NK activity in FC-lesioned animals did not differ from normals.

Previous experiments evaluating the effects of AHT lesions on mitogen-induced lymphocyte proliferation have shown that the maximum effect occurs 4 days after lesioning and returns to normal values by the 14th day (Cross *et al.*, 1980). Figure 2 shows that AHT lesions significantly ($P < 0{\cdot}01$) depress NK activity at 4 and 7 days. NK activity returns to normal values by the fourteenth day. Although NK activity is enhanced at day 14, it is not significantly different from that seen in normal animals. Hence the kinetics of the impairment of NK activity after AHT lesioning is similar to that observed when assessing the effects of AHT lesions on mitogen-induced lymphocyte proliferation.

Effect of glass bead column fractionation on NK activity

To further evaluate the effects of AHT lesions on NK activity, spleen cells from AHT, FC, or normal animals were fractionated on glass bead columns.

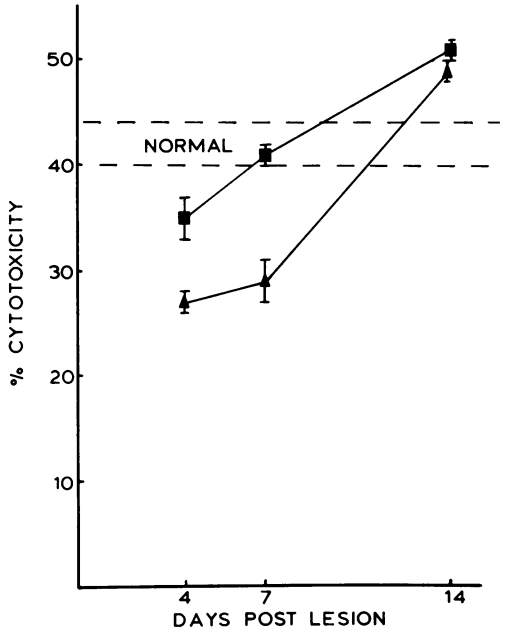

Figure 2. Splenic NK activity in AHT (▲), FC (■) and normal animals 4, 7 and 14 days after lesion placement (values represent 100:1 effector:target cell ratio). NK activity in AHT-lesioned animals is significantly different from FC-lesioned animals ($P < 0.01$) at both days, 4 and 7. NK activity in AHT- or FC-lesioned animals at day 14 did not differ from normal.

After fractionation, GBNA and GBA cell populations were tested for NK activity. NK activity is detected in all cell fractions. As shown in Table 1, AHT lesions impair NK activity in unfractionated spleen cell populations as compared to FC-lesioned or normal animals. NK activity in the GBNA cell fraction obtained from AHT, FC and normal animals do not differ. However, NK activity in the GBA cell fraction of the AHT-lesioned group is decreased when compared to that obtained from either FC-lesioned or normal animals. These data indicate that AHT lesions affect NK activity only in the GBA cell population.

Recent studies using a long-term 18 hr ^{51}Cr-release assay have shown that natural cytotoxic activity in rats may be due to cytotoxic macrophage activity (Brooks *et al.*, 1980). To investigate this, GBA spleen cells from AHT, FC, and normal animals were treated with carbonyl iron to remove macrophages. This treatment decreases the number of non-specific esterase positive cells from 12% to <1%. Removal of these macrophages (Table 1) did not alter the level of cytotoxicity within the GBA cell fraction of each group, indicating that cytotoxic macrophages are not involved in the lysis of F4 target cells. We have previously shown that AHT lesions enhance suppressor macrophage activity which is capable of inhibiting mitogen-induced lymphocyte proliferation (Roszman *et al.*, 1982). NK activity in AHT-lesioned animals continues to be impaired despite the removal of macrophages by carbonyl iron, thus, indicating that diminished NK activity does not result from enhanced macrophage suppression.

NK activity in hypophysectomized rats with AHT lesions

The next set of experiments were performed to determine the role of the pituitary gland in the

Table 1. NK activity of non-fractionated and glass bead column fractionated spleen cells from normal and FC-, AHT-lesioned animals*

Groups	Lytic units/10^6 cells ± SEM			
	Unfractionated	GBNA	GBA	Carbonyl iron-treated GBA
AHT Lesions ($n = 16$)‡	1·46 ± 0·06 ($P < 0.01$)†	1·59 ± 0·16 ($P > 0.05$)	1·42 ± 0·19 ($P < 0.05$)	1·45 ± 0·32 ($P < 0.05$)
FC Lesions ($n = 18$)	2·62 ± 0·62	1·61 ± 0·11	2·25 ± 0·25	2·75 ± 0·24
Normal ($n = 18$)	2·50 ± 0·30	1·71 ± 0·15	3·42 ± 0·67	2·86 ± 0·33

* Spleen cells obtained from animals 4 days after AHT or FC lesion placement.

† Significance was determined by Student's *t*-test comparing values obtained from lesioned animals to those from control animals. Normal and FC-lesioned animals did not differ significantly.

‡ n = number of animals/group.

Table 2. The effect of AHT lesions on NK activity in hypophysectimized animals*

	Lytic units/10^6 cells ± SEM	
Group	Non-hypophysectomized†	Hypophysectomized†
AHT	1·46 ± 0·06 (n = 16)‡	1·01 ± 0·06§ (n = 8)
FC	2·62 ± 0·62 (n = 18)	1·24 ± 0·08 (n = 10)
Normal	2·50 ± 0·30 (n = 18)	1·38 ± 0·13 (n = 6)

* NK activity determined 4 days after lesioning.

† NK activity in the hypophysectomized groups differs significantly from that of non-hypophysectomized groups ($P < 0·02$).

‡ n = number of animals/group.

§ NK activity did not differ significantly among the hypophysectomized groups.

suppression of NK activity after AHT lesioning. NK activity in all HX groups is significantly less than in animals with an intact pituitary (Table 2). These data indicate that hypophysectomy has a marked effect in impairing NK activity as previously reported (Saxena, Saxena & Adler, 1982). Furthermore, these observations suggest that pituitary factors are important for the induction of NK impairment by AHT lesions.

DISCUSSION

The most meaningful finding of this study is that electrolytic lesioning of the anterior hypothalamic area results in depressed NK activity. This is the first indication that discrete lesions in the hypothalamus affect the NK cell which may be important in immune surveillance. These data correlate with our previous reports on the effects of AHT lesions on mitogen-induced lymphocyte proliferation (Cross *et al.*, 1980; Brooks *et al.*, 1982). It is unlikely that the effect of AHT lesions on NK activity is the result of increased circulating corticosteroid levels because we have observed that both AHT and FC-lesioned animals have similar levels (Cross *et al.*, 1982). The transitory nature of these effects may result from either neuronal plasticity (Goldowitz, Scheff & Cotman, 1979) or the rapid turn-over of neurally controlled factors (e.g. hormones, peptides) which may serve as mediators of CNS modulation of immunity.

Recently Renoux *et al.* (1982) have shown that unilateral removal of the left neocortex impairs NK activity. Although small, discrete bilateral electrolytic lesions placed in the frontal cortex are associated with a slight decrease in NK activity, the suppression is not significant when compared to normal animals. These differences may result from the size and extent of the lesions or the method in which the lesions were produced (surgical *vs* electrolytic).

Previously we have demonstrated that AHT lesions enhance macrophage suppressor cell function (Roszman *et al.*, 1982). Hochman, Cudkowicz & Evans (1981) have shown that removal of macrophages by carbonyl iron increases splenic NK activity. The present report indicates that removal of macrophages by carbonyl iron does not alleviate the diminished NK activity resulting from AHT lesioning. Further experiments demonstrated that spleen cells can be fractionated to yield non-adherent and adherent cell fractions with NK activity. Interestingly, hypothalamic lesions employed in this study affect NK activity only in the adherent cell fraction, whereas activity in the non-adherent fraction is not altered. The diminished NK activity in the adherent fraction is not restored after removal of the macrophage. These findings indicate that macrophage suppression is not responsible for altered NK function. Moreover, in the present study, killing by the adherent population is not the result of macrophage-mediated cell lysis.

An intact pituitary is necessary for the maintenance of NK activity. Saxena, Saxena & Adler (1982) have shown that NK function is depressed in mice after hypophysectomy. Reconstitution with growth hormone restores NK activity to normal levels in these animals. Alternatively, Hanna & Schneider (1983) have shown that the steroid hormone, β-estradiol, suppresses NK activity. Recently, it has been shown that β-endorphins enhance NK activity in humans (J. E. Morley, personal communication). These studies lend support to the theory that hormones and neuropeptides modulate the NK cell. The present study confirms that hypophysectomy markedly decreases NK activity. Decreased NK activity in AHT lesioned animals is abrogated by hypophysectomy, suggesting that neural modulation of NK cells is affected by pituitary hormones or peptides. This conclusion must be tempered, however, by the observation that hypophysectomy has such a profound effect on NK activity that further modulation of NK function by non-pituitary factors (e.g. neurotransmitters) would be difficult to detect in this system. Further studies are needed to delineate the role of hormones, neurotransmitters and neuropeptides as modulators of NK cells.

In summary, the present study indicates that the

CNS, particularly the anterior hypothalamus, is capable of modulating the cytolytic activity of adherent NK cells. Thus, neuroimmunomodulation may be a potential factor in susceptibility to certain diseases.

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