Immunology 1987 **60** 461–465

Susceptibility of natural killer cell activity of old rats to stress

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Accepted for publication 6 November 1986

SUMMARY

We determined an *in vivo* response of NK cells in young and old rats towards the suppressive effect of stress. Stress was developed by isolating rats in separate cages, but control littermates were kept together. Animals were subjected to stress for 7 days, and alterations of NK cell activities were examined in the spleen, peripheral blood (PB) and bone marrow (BM). The results showed that old rats subjected to stress had a remarkable decrease in splenic and PB-NK activity compared to old control rats, concomitant with a highly increased level of NK cell activity in BM. Suppression of the lytic activity in the spleen of stressed old rats was correlated with a decrease in the percentage of conjugate formation between splenic NK cells and target tumour cells. In contrast, stressed young rats demonstrated relatively unchanged activity of NK cells examined in different tissues compared to age-matched controls. We concluded that old animals are more sensitive to the suppressive effect of stress compared to young ones, and the mechanism of this suppression is probably due to the migration of large granular lymphocytes (LGL) from spleen and PB to other sites such as BM.

INTRODUCTION

Several studies have demonstrated an intimate and interesting relationship between stress and the immune system. Stress is associated with alterations in humoral and cellular immune mechanisms in both laboratory animals and humans. For example, stress reduces lymphocyte-mediated cytotoxicity and decreases the response to mitogens and antigens (Sklar & Anisman, 1981; Keller *et al.*, 1981), reduces the level of circulating antibodies (Edwards & Dean, 1977), delays skin allograft rejection (Wistar & Hildemann, 1960) and reduces macrophage cytotoxicity (Pavlidis & Chirigos, 1980).

The mechanism by which stress suppresses different immune functions is not yet understood; however, it has been shown that exposure to stress can release opioid peptides from central and peripheral sites (Madden *et al.*, 1977; Shavit *et al.*, 1984) and corticosteroids (Ursin, 1980; De Souza & Van Loon, 1985). Thus, it is possible that opioid peptides and corticosteroids mediate some of the effects of stress on the immune system.

As a result, stressed animals become more susceptible to certain diseases including neoplasia. Different stressful states such as differential housing, restraint and sex-segregated groupings were found to modify the incidence of leukaemia (Ebbeson & Rask-Nielsen, 1967), sarcoma (Amkraut & Solomon, 1972) and mammary carcinoma (Newberry *et al.*, 1976; Henry, Stephens & Watson, 1975; Riley, 1975). Since natural cytotoxicity may be important in immunological surveillance against neoplasia and in the control of metastatic spreading of tumour cells (Herberman, 1983), the present study was undertaken to examine the effect of stress on the activity of natural killer (NK) cells in young and old rats because NK activity was found to be under age control (Ghoneum *et al.*, 1987; Itoh *et al.*, 1982).

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MATERIALS AND METHODS

Animals

Three- and 12-month-old Spraque–Dawley Albino male rats were obtained from the Animal Laboratory, University of California, Berkeley. Animals were provided with free access to food and water and were maintained in a 12-hr light cycle.

Stress induction

Stress was induced in rats according to the method previously described by Freidman, Glasgow & Ader (1969) with slight modifications. In brief, animals were grouped together for 1 week, then stress was induced by isolating animals in separate cages (one animal per cage) for 7 days. Cages were placed at a distance of not less than 2 m in each direction from each other to prevent the possible contact of isolated animals through smell. Control littermates were placed two animals per cage.

Naloxone treatment

Naloxone (Nal), which is known as a specific opiate antagonist

blocker, was used to indicate whether opioid stress was induced or not. Animals were given daily injections (0·1 mg/kg intraperitoneally) of naloxone hydrochloride (obtained from Du Pont Pharamaceuticals Inc., Manati, PR).

Experimental protocol

Animals were divided into five groups (G1–5). Each group consisted of four animals. The arrangement of the groups were as follows: G1 was stressed; G2 was stressed and given daily injections of Nal; G3, G4, and G5 served as controls; G3 was injected with Nal; G4 was injected with saline, and G5 received no injection. This protocol was applied for both young and old animals. The data represent mean ± SD of three different experiments carried out at different times, representing 12 animals/group and 120 in total.

Animal death

All animals were anaesthetized with ether before killing. Rats were kept for less than 1 min in a bell jar. Anaesthesia was employed by exposing the animals to ether via inhalation.

Preparation of lymphoid cells

Four animals in each group were killed 7 days after developing stress. Spleen, peripheral blood leucocytes (PBL) and bone marrow (BM) were prepared as previously described (Ortiz del Landazuri & Herberman, 1972; Oehler *et al.*, 1977; Ghoneum *et al.*, 1987) as follows.

Preparation of splenic lymphoid cells. Spleens were removed and teased in RPMI-1640 and passed through a 40-mesh stainless steel screen. Single cell suspensions from each group were pooled and cells were collected by centrifugation at 500 ***g***. Contaminating red blood cells were removed by suspending individual cell pellets in 1 ml of a lysing solution (8·25 g ammonium chloride, 1 g potassium chloride and 0·037 g tetrasodium EDTA dissolved in 1000 ml H_2O) for 1 min. Following incubation, cells were washed twice with Hanks' balanced salt solution (HBSS) and resuspended to a concentration of 10×10^6 cells/ml in complete medium (CM).

Isolation PBL. PBL were prepared from the fresh heparnized blood of rats by Ficoll–Hypaque density gradient. In brief, venous blood was drawn into large heparnized centrifuge tubes. The blood was diluted with an equal volume of HBSS, layered over a 10 ml of Ficoll, and centrifuged for 45 min at 400 ***g***. Cells removed from the interface were washed twice with HBSS. Cells of the same group were pooled and resuspended at a concentration of 10×10^6 lymphocytes/ml CM.

Isolation of bone marrow lymphoid cells. Marrow was obtained by flushing the femurs and tibiofibulas of rats with HBSS using a 10-ml syringe. Marrows of four animals were pooled and single-cell suspensions of marrow were prepared by repeated flushing using the same syringe. Cells were washed once by centrifugation prior to use.

Complete medium (CM)

This consisted of RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 100 U penicillin and 100 μg/ml streptomycin.

^{51}Cr-release cytotoxicity assay

Cultured YAC-1 target cells (5×10^6) were labelled with 100 μCi of sodium-51 chromate solution for 1 hr in 1 ml CM. After washing three times in 5 ml CM, 1×10^4 cells in 0·1 ml CM were pipetted into 96-well round-bottomed Linbro plates (Linbro Chemical Co., Hamden, CT). Previously washed effector cells were pipetted into quadruplicate wells to give effector : target cell ratios of 100:1, 50:1 and 25:1. Following a 4-hr incubation at 37°, 0·1 ml of supernatant from each well was collected and counted in a gamma counter. The percentage of isotope released was calculated by the following formula:

$$\% \text{ lysis} = \frac{\text{c.p.m. exp. release} - \text{c.p.m. spontaneous release}}{\text{c.p.m. total release} - \text{c.p.m. spontaneous release}} \times 100.$$

Target cells

YAC-1 cells, a Moloney leukaemia virus-induced T-cell lymphoma of A/Sn origin, were maintained in suspension culture at a starting density of 3×10^5/ml CM.

Effector target cell conjugate assay

The capacity of splenic NK effector cells from stressed and control groups to form conjugates with YAC-1 target cells were measured by the modification of a procedure described previously (Kumagai *et al.*, 1982). Briefly, 1×10^5 Percoll-isolated NK cells were incubated with 5×10^5 YAC-1 target cells in 1·0 ml CM in 12 × 75 mm glass tubes, pelleted at 130 ***g*** for 10 min, and incubated for 1 hr at 4°. Pellets were gently resuspended, and cytocentrifuged smears were prepared and stained with Giemsa. The percentage of conjugates was determined by counting 200 lymphocytes (bound and free) in triplicate samples.

Statistical analysis

A two-tailed Student's *t*-test was used to determine the significance of difference between stressed and control groups.

RESULTS

Effect of stress on splenic NK activity

As shown in Fig. 1, the ability of spleen cells to lyse YAC-1 lymphoma cells varies widely between young and old stressed

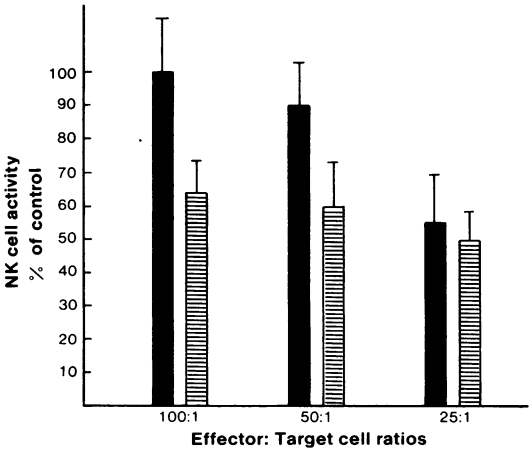

Figure 1. Comparison of splenic NK activity in young (■) and old (■) stressed rats. The data are expressed as a percentage of age-matched controls. The data represent mean ± SD of three experiments ($P < 0.005$).

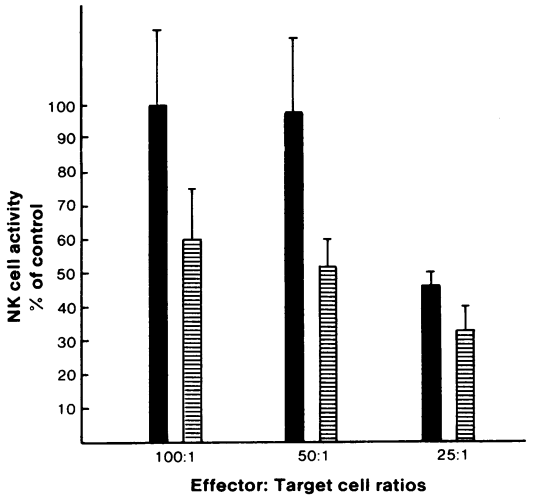

Figure 2. The effect of stress on the activity of NK cells from non-adherent peripheral blood mononuclear cells of young (■) and old (■) rats subjected to stress. The data are expressed as a percentage of age-matched controls. The data represent mean ± SD of three experiments ($P < 0.005$).

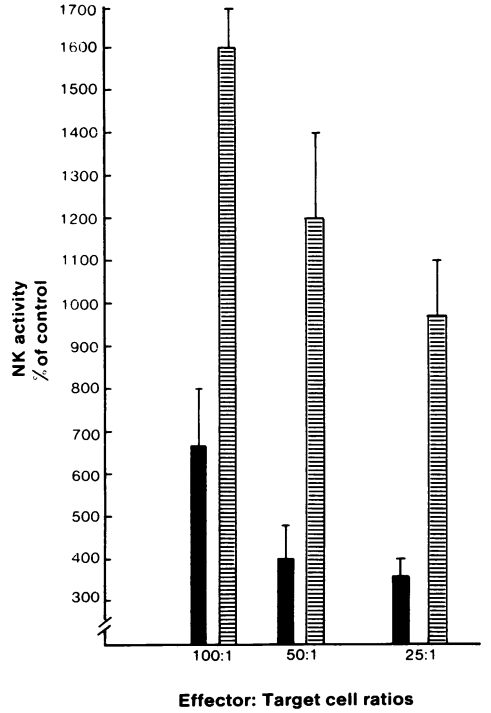

Figure 3. NK cell activity from the bone marrow of young (■) and old (■) rats subjected to stress. The data represented were determined as a percentage of age-matched controls. The data represent mean ± SD of three experiments ($P < 0.001$).

rats. Young stressed animals showed a slight suppression in splenic NK activity compared to young control. On the other hand, old rats appeared highly susceptible to the inhibitory effect of stress, showing 35–50% inhibition compared to old control.

Effect of stress on NK cell activity of peripheral blood (PB)

Results of the analysis of NK activities of the PB from stressed young and old subjects are shown in Fig. 2. It was also found that stress slightly decreased the cytotoxic reactivity of NK cells from PB of young rats. In contrast, old rats were more susceptible to the suppressive effect of stress where NK activity was significantly inhibited (40–65% of old control).

Effect of stress on NK activity of bone marrow (BM)

In contrast to spleen and PB, there was a highly significant elevation in the activity of NK cells in BM of stressed rats (Fig. 3). Interestingly, aged rats, when subjected to stress, demonstrated a higher level of NK activity than stressed young rats. The increase in NK activity of aged stressed rats was calculated approximately to be nine- to 16-fold than of old rat controls, whereas NK activity was elevated only by three to five-fold in young stressed rats in comparison to young rat controls.

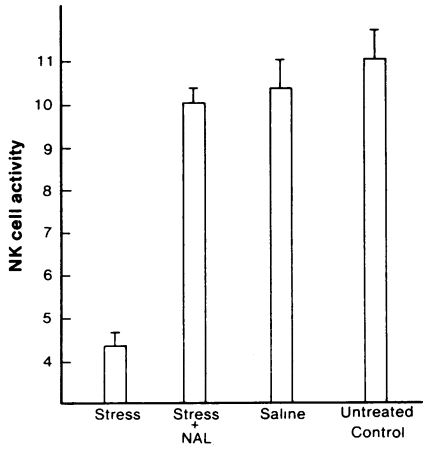

Figure 4. Reversal of stress-induced suppression of NK activity by naloxone. Naloxone was injected daily for 7 days during induction of stress. Control rats either received saline or were left untreated. The data represent mean ± SD of three experiments ($P < 0.001$).

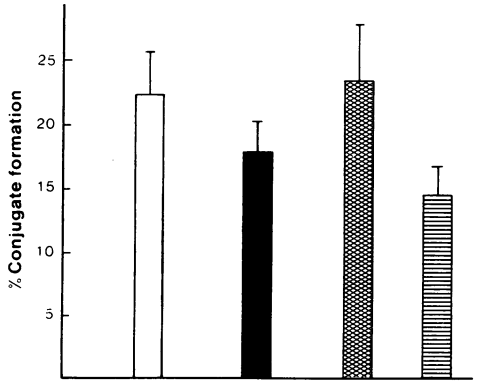

Figure 5. Effect of stress on conjugate formation between effector and target tumour cells. Splenic effector cells were isolated from spleens of young control (□), young stressed (■), old control (▩), and old stressed (■) rats. Conjugate formation was determined by counting 200 free and conjugated cells in cytospin preparation. The data represent mean ± SD of three experiments ($P < 0.0005$).

Reverse of stress effect by naloxone treatment

Data presented in Fig. 4 show that suppression in splenic NK activity in stressed old animals was reversed by naloxone administration. NK activity in rats treated with naloxone during the induction of stress was within control value. Neither naloxone nor saline injection affected NK activity compared to untreated controls.

Percentage conjugate formation

As shown in Fig. 5, the percentage conjugate formation between splenic effector cells and YAC-1 targets was decreased in stressed old animals (35% of old controls), while it was slightly decreased in young stressed rats (18% of young controls).

DISCUSSION

Many attempts have been made to evaluate the role of stress on NK activity. The results showed that the activity of NK cells is markedly reduced in animals by stressors such as electric shock (Shavit *et al.*, 1984), immersion in cold water (Aarstad, Gaudernack & Seljelid, 1984) and surgery, starvation, and transportation (Locke, 1982).

Many hypotheses have been postulated to analyse the mechanism of the suppressive effect of stress on NK cells. Aarstad *et al.* (1984) suggested that stress induces an increase of the corticosteroid (CS) level, which may directly suppress NK activity. It has been shown that a wide variety of stressors are associated with the rapid release of ACTH, followed by the release of CS (Burchfield, 1980). These CS have a suppressive effect on NK activity (Oehler & Herberman, 1978). Our previous study (Ghoneum *et al.*, 1986) demonstrated that the suppressive effect of CS depends on several factors, such as the nature of the hormone, dose, and time of treatment. Other mechanisms that may be involved in the suppression of NK activity by stress are opioids and morphine. Shavit *et al.* (1984) reported that opioid peptides and morphine released by stress might suppress NK activity directly or indirectly by modulating the release of certain hormones such as adrenocorticotropic hormone and adrenocorticosteroids. These hormones are known to suppress NK activity (Mathews *et al.*, 1983).

Data in the present study demonstrated that old animals subjected to stress had marked inhibition in cytotoxicity of splenic NK cells and a decrease in their binding capacity to target cells. This suppression is blocked by naloxone treatment. Similar findings have been reported by Shavit *et al.* (1984) using the opioid blocker naltrexone. It is reasonable to assume that suppression in the activity of NK in the spleen and PB, in conjunction with their increased activity in BM, further supports the idea that stress may cause redistribution of lymphocytes between different tissues. The phenomenon of lymphocyte redistribution has been reported in different animal models treated with CS. These include mice (Spry, 1972), rats (Berney, 1974; Mansour & Nelson, 1978) and guinea-pigs (Fauci, 1975). It also has been postulated to occur in man (Fauci, 1979). Cohen (1972) found that lymphopenia following an injection of glucocorticoid is not, as has been thought, due to lymphocyte destruction, but results from a process of lymphocytes leaving the blood and sequestering in tissues, especially in the bone marrow. According to Cohen, these lymphocytes remain in the bone marrow for a few days until GCC levels fall to normal, then they re-enter the circulation. Cohen & Crnic (1985) found a similar thing in T-cell migration into bone marrow in stressed mice. Earlier studies by Kiessling & Wigzell (1979) demonstrated that NK cells are developed from bone marrow; however, their activity is nearly absent in this tissue.

The age of the animal subjected to stress is a highly relevant factor. Data in the present study showed that old male Spraque–Dawley Albino rats are more susceptible to the suppressive effect of stress compared to young rats. It has been observed, in old animals, that the number and/or activity of suppressor cells was increased and the number and/or functional efficiencies of immunocompetent cells decreased (Makinodan *et al.*, 1976). In addition, other various immunological functions and responses are affected by the ageing process.

Experiments carried out by us (Ghoneum & Egami, 1982) and others (Tsang & Fudenberg, 1985) demonstrated that thymocyte numbers, responsiveness to thymus-dependent antigens, monocyte chemotaxis and lymphocyte response to PHA decrease with advancing age. Similarly, NK cells showed an age-related decline in their activity in mice (Ghoneum *et al.*, 1987; Itoh *et al.*, 1982) and rats (Reynolds & Holden, 1982). Bloom (1985) suggested that, since the functions of old lymphocytes are already diminished and their regulatory systems are shifted, they might be more vulnerable to the effect of stress. She also suggested that the changes in endocrine function could alter the response to stress of the immune system with age.

ACKNOWLEDGMENTS

We are indebted to Dr L. King, Associate Dean for Research, and Dr C. Thomas, Chairman, Department of Psychiatry at Drew Medical School, for their time and for encouraging the work related to studies of stress. The expert animal care of Dr J. Brown and R. Williams is gratefully acknowledged. We would also like to acknowledge the technical assistance of S. Kim.

REFERENCES

Aarstad H.J., Gaudernack G. & Seljelid R. (1984) Stress causes reduced natural killer activity in mice. *Scand. J. Immunol.* **18,** 461.

Amkraut A. & Solomon G.F. (1972) Stress and murine sarcoma virus (Moloney) induced tumors. *Cancer Res.* **32,** 1428.

Berney S. (1974) Characterization of glucocorticosteroid-induced changes in lymphocyte circulation in the rat. *Clin. Res.* **22,** 414A.

Bloom E.T. (1985) The aging lymphocyte, stress and immunity. In: *Stress, Immunity and Aging* (ed. E. L. Cooper), p. 135. Marcel Dekker Inc., New York.

Burchfield S.R. (1980) Pituitary adrenocortical response to chronic intermittent stress. *Physiol. Behav.* **24,** 297.

Cohen J.J. (1972) Thymus-denied lymphocytes sequestered in bone marrow of hydrocortisone treated mice. *J. Immunol.* **108,** 841.

Cohen J.J. & Crnic L.S. (1985) Behavior, stress, and lymphocyte recirculation. In: *Stress, Immunity and Aging* (ed. E. L. Cooper), p. 73. Marcel Dekker Inc., New York.

De Souza E.B. & Van Loon G.R. (1985) Differential plasma B-endorphin, B-lipotropin and adrenocortiotropin responses to stress in rats. *Endocrinology*, **116,** 1577.

Ebbeson P. & Rask-Nielsen R. (1967) Influence of sex-segregated grouping and of inoculation with subcellular leukemic material on development of nonleukemic lesions in DBA/2, BALB/c and CBA mice. *J. natl. Cancer Inst.* **39,** 917.

Edwards E.A. & Dean L.M. (1977) Effects of crowding of mice on

humoral antibody formation and protection to lethal antigenic challenge. *Psychosom. Med.* **39,** 19.

FAUCI A.S. (1975) Corticosteroids and circulating lymphocytes. *Transplant. Proc.* **7,** 37.

FAUCI A.S. (1979) Immunosuppressive and anti-inflammatory effects of glucocorticoids. *Monogr. Endocrinol.* **12,** 449.

FREIDMAN S.B., GLASGOW L.A. & ADER R. (1969) Psychosocial factors modifying host resistance to experimental infections. *Ann. NY Acad. Sci.* **164,** 381.

GHONEUM M.M.H. & EGAMI N. (1982) Age related changes in morphology of the thymus of the fish *Orgzies latipes*. *Exp. Geront.* **17,** 33.

GHONEUM M., EGAMI N., IJIRI K. & COOPER E. (1986) Effect of corticosteroids on the thymus of the fish *Oryzias latipes*. *J. Dev. Comp. Immunol.* **10,** 35.

GHONEUM M., GILL G., WOJDANI A., PAYNE C. & ALFRED L. (1987) Suppression of basal and *Corynebacterium parvum*-augmented NK activity during chemically induced tumour development. *Int. J. Immunopharm.* (in press).

HENRY J.P., STEPHENS P.M. & WATSON F. (1975) Force breeding, social disorder and mammary tumor formation in CBA/USC mouse colonies: a pilot study. *Psychosom. Med.* **37,** 277.

HERBERMAN R.B. (1983) Possible role of natural killer cells in host resistance against tumors and other diseases. *Clin. Immunol. Allergy,* **3,** 479.

ITOH K., SUZUKI R., UMEZU Y., HANAUMI K. & KUMAGAI K. (1982) Studies of murine large granular lymphocytes. II. Tissue, strain and age distribution of LGL and LAL. *J. Immunol.* **129,** 395.

KELLER S.E., WEISS J.M., SCHEIFER S.J., MILLER N.E. & STEIN M. (1981) Suppression of immunity by stress: effect of a graded series of stressors on lymphocyte stimulation in the rat. *Science,* **213,** 1397.

KIESSLING R. & WIGZELL H. (1979) An analysis of the murine NK cells as to structure function and biological relevance. *Immunol. Rev.* **44,** 165.

KUMAGAI K., ITOH K., SUZUKI R., HINUMA S. & SAITOH F. (1982) Studies of murine granular lymphocytes. I. Identification as effector cells in NK and K cytotoxicities. *J. Immunol.* **129,** 388.

LOCKE S.E. (1982) Stress, adaptation and immunity: studies in humans. *Gen. Hosp. Psychiatry,* **4,** 49.

MADDEN J., AKIL H., PATRICK R.L. & BARCHAS J.D. (1977) Stress-induced parallel changes in central opioid levels and pain responsiveness in the rat. *Nature (Lond.),* **265,** 358.

MAKINODAN T., ALBRIGHT J.W., GOOD R.I., PETER C.P. & HEIDRICK M.L. (1976) Reduced humoral immune activity in long-lived old mice: an approach elucidating its mechanism. *Immunology,* **31,** 903.

MANSOUR A. & NELSON D.S. (1978) Effect of hydrocortisone on the response of rat lymphocytes to phytohemagglutinin. *Austr. J. exp. Biol. Med. Sci.* **56,** 301.

MATHEWS P.M., FROELICH J.C., SIBBIT W.L., & BANKHURST A.D. (1983) Enhancement of natural cytotoxicity by β-endorphin. *J. Immunol.* **130,** 1658.

NEWBERRY B.H., GILDOW J., WOGAN J. & REESE R.I. (1976) Inhibition of huggins tumors by force restraint. *Psychosom. Med.* **38,** 155.

OEHLER J.R. & HERBERMAN R.B. (1978) Natural cell-mediated cytotoxicity in rats. III. Effects of immunopharmacologic treatments on natural reactivity augmented by polyinosinic-polycytidylic acid. *Int. J. Cancer,* **21,** 221.

OEHLER J.R., HERBERMEN R.B., CAMPBELL D.A. & DJEY J.Y. (1977) Inhibition of rat mixed lymphocyte cultures by suppressor macrophages. *Cell. Immunol.* **29,** 238.

ORTIZ DEL LANDAZURI M. & HERBERMAN R.B. (1972) Immune response to gross virus induced lymphoma. Three characteristics of the cellular immune response. *J. natl. Cancer Inst.* **49,** 147.

PAVLIDIS N. & CHIRIGOS M. (1980) Stress-induced impairment of macrophage tumoricidal function. *Psychosom. Med.* **42,** 47.

REYNOLDS C.W. & HOLDEN H.T. (1982) Genetic variation in natural killer (NK) activity in the rat. In: *NK Cells and Other Natural Effector Cells* (ed. R. B. Herberman), p. 319. New York.

RILEY V. (1975) Mouse mammary tumors: alteration of incidence as apparent function of stress. *Science,* **189,** 465.

SHAVIT Y., LEWIS J.W., TERMAN G.W., GALE R.P. & LIEBESKIND J.C. (1984) Opioid peptides mediate the suppressive effect of stress on natural killer cell cytotoxicity. *Science,* **223,** 188.

SKLAR L.S. & ANISMAN H. (1981) Stress and cancer. *Psychol. Bull.* **89,** 369.

SPRY C. (1972) Inhibition of lymphocyte recirculation by stress and corticotropin. *Cell. Immunol.* **4,** 86.

TSANG K. & FUDENBERG H. (1985) Restoration of immune responses in aging animal models. In: *Stress, Immunity and Aging* (ed. E. L. Cooper), p. 257. Marcel Dekker Inc., New York.

URSIN H. (1980) Personality, activation and somatic health, a new psychosomatic theory. In: *Coping and Health* (eds S. Levine and H. Ursin), p. 259. Plenum Publishing Corp., New York.

WISTER R. & HILDEMANN W.H. (1960) Effect of stress on skin transplantation immunity in mice. *Science,* **131,** 159.