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Modulation of Behaviour and Testosterone Concentration in Immunodepressed Male Laboratory *Mice (Mus musculus)*

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BARNARD, C. J., J. M. BEHNKE, A. R. GAGE, H. BROWN AND P. R. SMITHURST. *Modulation of behaviour and testosterone concentration in immunodepressed male laboratory mice* (Mus musculus). PHYSIOL BEHAV **61**(6) 907–917, 1997.—Recent ideas suggest that current immunocompetence may act as a constraint on behavioural and physiological decisions, where these risk imposing an additional burden on immune function. We tested this in the context of time budgeting and the secretion of the potentially immunodepressive hormones testosterone and corticosterone, by treating adult male CFLP laboratory mice with anti-thymocyte serum (ATS) to depress thymus-mediated immune function. In comparison with males given a naive rabbit serum (NRS) vehicle control, ATS-treated mice showed a reduction in serum testosterone concentration, aggressive behaviour, and general activity, and maintained time spent sleeping, relative to pretreatment levels. Behaviours that differed between treatments correlated with measures of immunodepression (reduction in relative thymus weight or serum total IgG concentration), but relationships with behavioural changes were independent of those with testosterone. There was little evidence that changes were affected by social status. The results are discussed in the context of the adaptive modulation of immune function and physiological and behavioural decision-making. © 1997 Elsevier Science Inc.

Testosterone Mice Immunodepression Behaviour Corticosterone Modulation

RECENT discussions have suggested that immune function has an important influence on physiological and behavioural decision-making [e.g., (6,14,26,32,35)]. In particular, several hormones and cytokines from the gonads and adrenal, thyroid, thymus, pituitary, and pineal glands interact directly or indirectly with the immune system [e.g., (1,14,30,31)], with the effect that some components of the immune response may become down-regulated. Relationships between immunodepression and the secretion of androgens and glucocorticoids have been appreciated for some time [(1,13,15,16,28) but see e.g., (7,31) for evidence of other immunomodulatory effects], and there is evidence that feedback mechanisms operate to modulate the secretion of hormones as immune status varies (15). More recently, such mechanisms have been viewed as an element in the adaptive modulation of hormone secretion, behaviour, and morphological (particularly secondary sexual) characters, where these have consequences for immunocompetence [(5,14,32,35), but see (27)]. In this view, immunocompetence may be conserved (by down-regulating hormone secretion) and, thus, act as a constraint on other categories of decision-making (e.g., sexual display), or immunodepression may be tolerated and reflect an adaptive life history decision to shift metabolic resources between physiological systems, with immunodepressive hormones providing part of the mechanism for regulating investment in immune function (5,32,35).

In a series of experiments with male laboratory mice of the CFLP strain, we have shown that aggressive behaviour and serum hormone (testosterone and corticosterone) and total IgG [a bystander measure of immunocompetence (4–6)] concentrations covary in ways that are consistent with the adaptive modulation hypothesis and influence resistance to an experimental infection of the piroplasmid protozoan *Babesia microti* (3–6,32). Moreover, interrelationships differ between males of different social status and between social/sexual contexts in accordance with rank-related modulation of hormone secretion and an adaptive trade-off between immunocompetence and apparent reproductive opportunity (2,5,32).

So far, we have examined these interrelationships only as they occur spontaneously within individuals. If hormone concentrations and behaviour are modulated in response to current immune status, however, we should be able to manipulate immunocompetence experimentally and predict resultant hormonal and be-

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havioural changes. A consistent finding in our previous work has been that, although increased aggressive behaviour and testosterone concentration were both associated with reduced immunocompetence and resistance to *B. microti*, they were not themselves significantly correlated (5,6,32). This suggests that hormonal and behavioural changes may provide independent avenues of response to variation in immune status, rather than behaviour reflecting changes in underlying hormonal causal mechanisms [e.g., (36)].

To test our hypothesis, an immunodepressive procedure was required that would induce significant temporary immunodepression while causing minimal undesirable side effects. For this reason, cytotoxic drugs (e.g., corticosteroids), whole-body irradiation, and ablation of primary lymphoid organs by surgery were inappropriate. Treatment with heterologous antithymocyte serum (ATS) was selected because the technique is long-established, relatively innocuous, and without the general side effects of other forms of immunodepressive therapy (22,23), and acts primarily on T-lymphocytes, the helper cells that are essential for efficient antibody responses and cell-mediated immunity [(12,23,34) see (22,33) for full reviews]. In addition, there are known feedback mechanisms mediating the secretion of sex steroids in relation to immunocompetence via the thymus (1,15). In potentially lowering all circulating T cells in the host, ATS treatment was preferred to treatment with monoclonal antibodies that would have required the administration of 2 reagents (antiCD4 and antiCD8) and suitable controls to achieve the same end. In this experiment, therefore, we compared changes in behaviour and serum testosterone and corticosterone concentrations in mice treated with rabbit (*Oryctolagus cuniculus*) antimouse thymocyte serum with that of mice treated with serum from naive rabbits (NRS). It is important to emphasize that the experiment is restricted to testing the high level associations between behaviour and hormonal relationships with immunocompetence predicted by our previous experiments. It acknowledges, but is not concerned with, the complex mechanisms and metabolic pathways that are known to underlie these associations (14,16,21,30,31). Although feedback from thymus activity is only one route by which immune function appears to influence testosterone levels (1,14,15), we predicted a decline in serum testosterone concentration in ATS-treated animals. Because the degree of modulation of testosterone has consistently been associated with immune function and resistance in our previous experiments [e.g., (5,32)], this provided an opportunity to test for the independent effects of reduced immunocompetence on testosterone concentration and behaviour inferred above. We did not make any prediction regarding corticosterone concentration because evidence for modulation of corticosterone relative to immune status in our experiments is weak [(5,32), but see (4)]. Our prediction with respect to behaviour was that immunodepressive treatment would reduce the performance of potentially stressful activity, such as aggression, and possibly other metabolically demanding behaviours like running and climbing and, at the same time, increase the incidence of less demanding behaviours, such as sleeping. If aggression was reduced, we might also expect an increase in social investigation, because a reduction in aggression may destabilize aggressive social relationships and lead to an increase in information-seeking [e.g., (17,18)].

In our previous work, high- and low-ranking males differed in their tendency to modulate testosterone concentration, with the result that testosterone-related resistance to *B. microti* was limited to those of high rank. In the present experiment, therefore, we tested for an interaction between ATS/NRS treatment and social rank in the tendency to modulate hormone concentrations and behaviour.

METHODS

Pre-Experimental Procedure

The subjects were 64 male laboratory mice of the randomly bred CFLP strain (3) purchased from Bantin and King Ltd, Hull, UK. At 42 days of age, groups of 4 animals were established in polypropylene cages (12.5 × 45 × 14 cm) for 3 weeks to acclimatize to laboratory conditions. From purchase, and throughout the experiment, animals were maintained on a 12 h:12 h reversed light:dark cycle (lights on at 2000 h, lights off at 0800 h). Food (Harlan Tetrad TRM rat/mouse diet) and water were provided ad lib. At 60 days of age, mice were weighed, given individually distinctive fur marks using Clairol 'Nice 'n' Easy™, Natural Black' hair dye (Bristol Myers Ltd, Uxbridge, UK) and an 88 μl sample of blood was taken from each animal using the tail-sampling procedure of Smith et al. (32).

Pretreatment Groups

Three days after the pre-experimental blood samples were taken, the mice were re-allocated arbitrarily to 16 groups of 4 individuals (cage dimensions as for pre-experimental groups), ensuring that no males within a group had previously encountered each other. Mice remained in their pretreatment groups for 8 days, during which the amount of time spent in different behaviours was recorded in 2 ways, following the methods of Hurst et al. (19) and the behaviour categories of Mackintosh (24) and Kareem and Barnard (20). First, all social and nonsocial behaviours (Table 1) performed by each individual were recorded using instantaneous spot-checks. Observations of groups (totalling 21 h) were randomized through the 12h dark phase and carried out under dim red illumination (3). Each mouse was observed as a focal animal for a total of 160 spot-checks over the 8 days, during which the behaviour being performed at the moment of observation was recorded on a check sheet. Because social behaviours, in particular, tend to be brief, they are likely to be underrepresented in spot-check samples (19). In addition, therefore, groups were observed continuously for 2 5-min periods per day each (a total of 16 h observation, again randomized through the dark phase) to record the social behaviours initiated and received by each individual, and the identity of the other mice with which it interacted. In the case of each aggressive interaction, the degree of escalation involved was recorded on an arbitrary scale ascending from 1 (threat, usually with no physical contact) to, rarely, 5 (escalated aggression involving biting and chasing) (see Table 1).

Treatment Procedure

At the end of the 8-day pretreatment period, mice were weighed again and a second 88 μl blood sample taken from the tail. Pretreatment groups were then allocated randomly to 1 of 2 treatment batches (8 groups each), and mice were separated and housed singly for 6 days in the same sized cages as pretreatment groups.

On the day following separation, 8 groups (32 mice) were injected IP with 0.5 ml ATS (ATS-treated mice) and the remaining 8 groups were injected with 0.5 ml of NRS (NRS control mice). These injections were repeated on days 2 and 4 of separation, except that the volume of serum given was halved on day 2 (0.25 ml). Two further blood samples were taken. A 20-μl sample was taken from the tail 1 day after the first ATS/NRS injection for white blood cell counts. A 50 μl retro-orbital sample, following Barnard et al. (3) was then taken on day 5 (i.e., 1 day after the third injection of ATS/NRS). On the final day of treatment, animals were again weighed and all individuals were

TABLE 1

BEHAVIOUR CATEGORIES RECORDED DURING THE EXPERIMENT

Social investigation: a composite category combining the olfactory investigatory behaviours Sniff, Nose and Body (sensu 18, 21)
Aggression: a composite category combining Offensive upright, Offensive sideways, Threat, Circling, Bite, and Chase (sensu 18, 21)
Defensive behaviours: a composite category combining Freeze, Flee, Defensive sideways, and Defensive upright (sensu 18, 21)
Allogroom: A mouths and licks the fur of B, mostly on the back and nape
Mount/Attempted mount: A mounts, or attempts to mount, B from the rear
Eat: Consumes food from the food hopper or elsewhere in the cage
Drink: Drinks from the water hopper
Sleep: lying or sitting unalert and eyes observed or presumed (when head of focal mouse obscured in huddle or under food hopper) to be closed
Groom: Grooms genitalia or other body parts, including washing of the face with forepaws
Mobile: Movement around the cage, including running and ambling
Sniff sawdust: sniffs the sawdust on the floor of the cage
Dig sawdust: Digs in the sawdust with forepaws or kicking sawdust with hind legs
Crouch: Crouches on hind quarters
Climb: Climbs up the side of the cage or on the wire lid of the cage with all feet off the ground
Sniff air: Sniffs the air, not directed towards any other animal or part of the cage
Sniff towards observer: Sniffs the air in direction of the observer, normally while climbing against the side of the cage
Upright scan: Stands upright on back legs, usually sniffs the air
Investigate cage: Sniffs the plastic sides of the cage, usually mobile
Jerk: Uncontrolled sudden movement from a standard position, sometimes flipping over completely or followed by fast running

injected with 0.2 ml of a sheep erythrocyte suspension (SRBC) containing 25×10^7 SRBC/ml (each mouse receiving 5×10^7 SRBC).

Preparation and Preliminary Evaluation of Antithymocyte Serum

Antimouse thymocyte serum was prepared by a modified procedure of Levey and Medawar [(23) but see (22) for a full review]. Adult female New Zealand white rabbits were injected with a thymocyte cell suspension prepared from 11 4-week-old male C57BL/10 mice (approximately $2-5 \times 10^8$ thymus cells/rabbit), using aseptic techniques as described for lymphocytes in Behnke and Parish (8), differing only in that cell suspensions were prepared in RPMI medium without foetal calf serum. Rabbits were injected intravenously into the lateral ear vein with the required number of cells in a volume not exceeding 0.9 ml. This procedure was repeated 2–3 weeks later and the rabbits were exsanguinated after a further 7 days. The serum was separated after clotting at 40°C, heat inactivated at 56°C for 45 min, aliquoted, and stored at −80°C until required. Note that thymocytes were obtained from a different mouse strain to the CFLPs used in the experiment to minimize cross-reactivity with tissue antigens other than those of T-lymphocytes. NRS was obtained from female New Zealand white rabbits of approximately the same age.

The efficacy of the ATS preparation in reducing thymocytes was confirmed through tests carried out prior to its use in the experiment. Clumping and lysis of target thymocytes were demonstrated in vitro in the presence of complement (1/200 final dilution of ATS caused 61% reduction in cells in the presence of 1/40 complement and 4.4×10^7 thymocytes from 4-week-old male CFLP mice).

In a pre-experimental trial evaluation of the in vivo consequences of injecting our ATS preparation, a group of 3 CFLP mice were injected IP either with ATS or the same volume of NRS (0.125, 0.25, and 0.5 ml, respectively). Peripheral white blood cell concentrations were measured a day before and after injection using standard techniques (11). Injection with ATS at all volumes caused a reduction in the concentration of peripheral white blood cells averaging 49.2%, whereas injection of NRS to 3 control mice resulted in a mean increase of 18%.

Posttreatment Groups

The day after injection with SRBCs, mice were re-established in their pretreatment groups, and behavioural observations repeated as for pretreatment groups for a further 8 days.

Organ Weights and Blood Assays

At the end of the posttreatment observation period, mice were weighed for the final time, killed using chloroform, and exsanguinated. The kidneys, adrenal glands, spleen, thymus gland, testes, preputial glands, seminal vesicles, heart, and mesenteric lymph nodes of each individual were carefully dissected out and weighed.

Haematology and Haemagglutination Assays

The packed cell volume (PCV) of blood samples was determined with a standard PCV reader after centrifugation of blood samples in capillary tubes in a haematocrit centrifuge.

Sheep erythrocytes (SRBC) were obtained fresh in Alsever's solution and washed 3 times in phosphate buffered saline (PBS). The concentration of cells was determined by standard haemocytometry, and the cells were resuspended to the required concentration in sterile PBS. For determination of the antibody response to SRBC, heat-inactivated (56°C for 45 min) mouse sera were titrated out by serial doubling dilution on plastic microtitre plates, the first well in each titration containing 40 μl of neat serum in PBS. A 3% (v/v) washed SRBC suspension in a volume of 20 μl was added to each well, the plates were left to stand at 37°C for 2 h and the endpoint for haemagglutination was read by visual inspection.

Measurement of Serum Hormone and IgG Concentrations

Pre-experimental, pretreatment, posttreatment, and terminal blood samples were assayed for serum concentrations of testosterone, corticosterone, and total IgG following Barnard et al. (4–6).

Testosterone. The concentration of testosterone (ng/ml) was measured using a Coat-a-Count™ solid-phase ^{125}I total testosterone kit (Diagnostic Products Corporation, Los Angeles, CA) with 25-μl samples of undiluted serum for experimental and cal-

ibration assays. Testosterone concentrations were calculated by reference to the calibration curves.

Corticosterone. The concentration of corticosterone (ng/ml) was measured using 6-μl samples of undiluted serum and a Gamma-B ^{125}I-corticosterone kit (Immunodiagnostic Systems Ltd, Los Angeles, CA) based on double antibody radioimmunoassay, as advised by the manufacturers. Corticosterone concentrations were calculated by reference to standards provided with the kit.

Total IgG. Serum total IgG concentration (mg/l) was determined by the method of Mancini et al. (25), using radial immunodiffusion kits (The Binding Site, Birmingham, UK). Ring diameters were measured in 2 directions at 90° and the mean was used to calculate the concentration of immunoglobulins from a calibration curve obtained using appropriate standards.

In a small number of cases, limited serum volumes meant it was not possible to obtain a reliable estimate of all 3 serum factors from a particular sample. As a result, sample sizes in some subsequent analyses vary [see also (4–6)].

Statistical Analyses

All analyses were performed using Statgraphics Plus™ version 7 (Manugistics Ltd, MD). Parametric analyses were used throughout (data were $\log_{10}$ or square root transformed as necessary and tested for normality using a Kolmogorov-Smirnov 1-sample test). Wherever there were a priori reasons for expecting trends or differences in a particular direction, probabilities associated with significance tests are indicated as 1-tailed.

RESULTS

Social Rank

Following Barnard et al. (4–6), high- and low-rank categories within groups were defined on the basis of the ratio of attacks initiated and received by each male during the pretreatment period of grouping. As in our previous experiments, high-ranking males were identifiable as having high initiation:receipt ratios and initiating significantly and disproportionately more attacks over the pretreatment period [$t(26) = 3.96$, $p < 0.001$]. Two groups (one from each treatment), in which the incidence of aggression was very low, were omitted from later analyses because it was not possible to allocate males to rank categories. All other groups comprised 1 high- and 3 low-ranking males. All analyses relating to social status were based on high- and low-rank categories and data for those of low rank were averaged within cages to control for potential problems of nonindependence (4,5).

Pre-Experimental Differences in Physiological Measures and Body Weight

To control for any chance pre-experimental differences in physiological measures between mice subsequently allocated to different treatments, we carried out a series of 2-way analyses of variance (ANOVA) with pre-experimental PCV and serum concentrations of testosterone, corticosterone, and total IgG as dependent variables and subsequent treatment and pretreatment rank category as factors. Pre-experimental body weight was included as a covariate. No significant pre-experimental bias emerged in any variable with respect to treatment or rank category. By chance, however, there was a significant difference in body weight between mice allocated to the 2 treatments, with mice allocated to ATS treatment being slightly, but significantly, heavier (mean ± SE pregrouping weight = 32.01 ± 0.27 g) than those designated as NRS controls (31.03 ± 0.33 g) [$F(1, 24) = 6.83$, $p < 0.02$]. Although there was no association between pre-experimental body weight and subsequent aggressiveness in pretreatment [$r(28) = -0.07$, NS] or posttreatment groups [$r(28) = 0.007$, NS], body weights relating to pre- and posttreatment phases were, nevertheless, taken into account when analyzing treatment effects on aggressive behaviour.

Effects of Treatment on Measures of Immunocompetence

If treatment with ATS depressed immunocompetence, we should expect an associated reduction in our measures of immune function.

Two-way ANOVA with treatment and pretreatment rank category as factors showed that, as expected, thymus weight (as % body weight) in ATS-treated animals was reduced relative to NRS controls [$F(1, 25) = 3.72$, 1-tailed $p < 0.05$, Fig. 1], implying loss of thymus cells as a result of treatment. There was no significant difference between rank categories in thymus weight or any interaction between treatment and rank category.

Depression of immune function was also confirmed by terminal sample haemagglutination responses to SRBCs. Two-way ANOVA revealed a significant reduction in haemagglutination titre in ATS-treated males [$F(1, 24) = 48.33$, 1-tailed $p < 0.0001$, Fig. 2]. There was no main effect or interaction with respect to rank category and no effect of terminal body weight (covariate).

In terms of intercurrent measures, ANOVA of the change in total IgG concentration across the period of separation and treatment (post- minus pretreatment concentration, controlling for change in body weight over the same period as a covariate) revealed a sharp decline in ATS-treated mice, compared with an

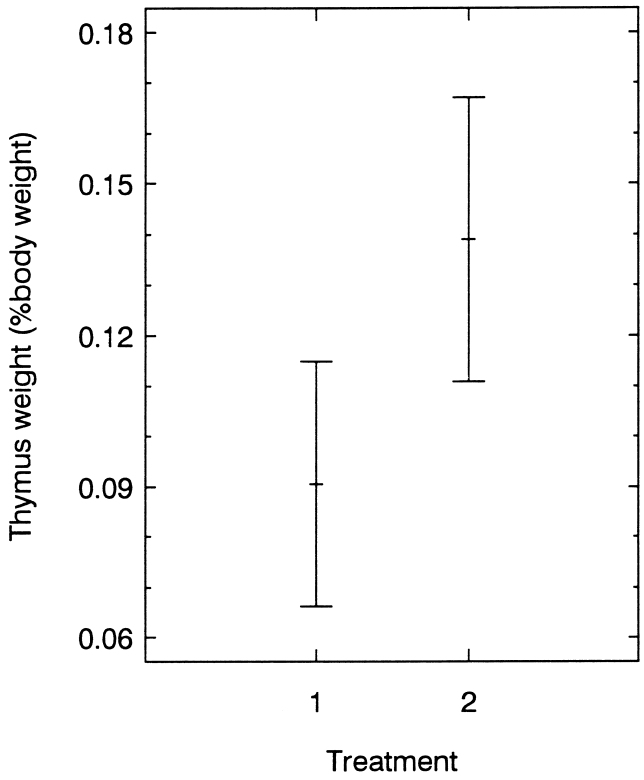

FIG. 1. Least-squares means from ANOVA of thymus weight (% terminal body weight) in ATS- (Treatment 1) and NRS- (Treatment 2) treated mice. Bars are least-squares deviations. See text.

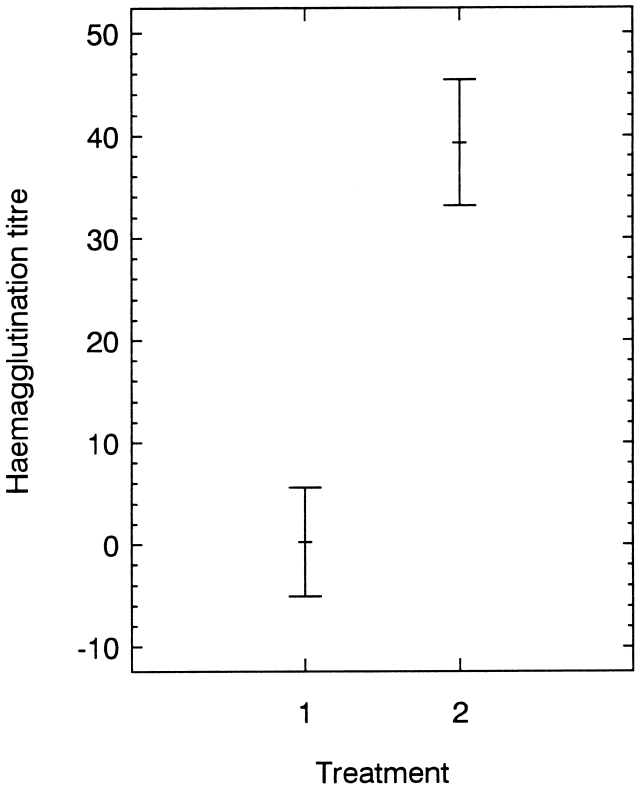

FIG. 2. Least-squares means from ANOVA of haemagglutination titre (1/dilution) in response to SRBC challenge in ATS- and NRS-treated mice. Treatments and bars as in Fig. 1. See text.

average increase among NRS-treated animals [$F(1, 23) = 20.78$, 1-tailed $p < 0.03$, Table 2]. There was also a significantly greater reduction in total IgG among pretreatment high-rankers compared with low-rankers [$F(1, 23) = 5.05$, $p < 0.04$], but no significant interaction between treatment and rank category. There was no significant change in body weight over the period of isolation and treatment. The greater decline among ATS-treated animals resulted in significantly lower posttreatment IgG concentrations compared with those treated with NRS [$F(1, 23) = 6.93$, 1-tailed $p < 0.01$, Table 2], but there was no difference or interaction with rank. ATS and NRS animals did not differ significantly in pretreatment IgG concentrations [$F(1, 23) = 0.06$, NS, Table 2].

Total IgG concentration increased significantly over the period of grouping in both ATS- and NRS-treated mice before and after isolation and treatment (Table 2, paired *t*-test, $p < 0.005$ in all cases). Two-way ANOVA of pretreatment change in IgG revealed no difference in the degree of increase between rank categories or mice subsequently subjected to different treatments. Although there were also no main effects of rank and treatment in posttreatment groups, there was a suggestive interaction between the two; as might be expected from previous rank differences in IgG response during grouping (in which those of high-rank showed a decline in IgG concentration (6), the posttreatment increase tended to be lower among those of high rank following ATS treatment [$F(1, 24) = 3.28$, $0.1 > P < 0.05$, Fig. 3].

Effect of Treatment on Serum Hormone Concentrations

If mice modulate testosterone secretion in relation to current immune status, we should expect a decline in serum concentration across the period of treatment in ATS-treated individuals. Figure 4 shows that this was the case [$F(1, 23) = 3.84$, 1-tailed $p < 0.05$], ATS-treated mice showing a reduction in serum concentration compared with a slight increase among NRS-treated animals. Because there was no significant difference between ATS- and NRS-treated mice in pretreatment testosterone concentration [$F(1, 23) = 0.008$, NS], the greater decline in ATS-treated animals resulted in significantly lower posttreatment concentrations [$F(1, 23) = 3.94$, 1-tailed $p < 0.05$]. There was no significant difference or interaction with rank and a significant effect of current body weight only in the case of pretreatment concentrations [$F(1, 23) = 4.51$, $p < 0.05$]. To relate the decline in testosterone concentration over the period of treatment to our measures of immunocompetence, we carried out a stepwise partial regression analysis with thymus weight (as % body weight),

TABLE 2

MEAN ± SE SERUM CONCENTRATIONS OF TOTAL IgG, TESTOSTERONE, AND CORTICOSTERONE FOR ATS-TREATED AND NRS CONTROL MICE AT EACH SAMPLING POINT DURING THE EXPERIMENT

		ATS	NRS
Total IgG (mg/l)	Pre-exp	1866 ± 122 (16)	1841 ± 89 (12)
	Pretrtmt	2755 ± 210 (16)	2708 ± 283 (12)
	Posttrtmt	2123 ± 217 (16)	2994 ± 209 (12)
	Termination	8748 ± 1128 (16)	8930 ± 528 (12)
Testosterone (ng/ml)	Pre-exp	14.4 ± 4.8 (16)	8.7 ± 3.2 (12)
	Pretrtmt	9.8 ± 2.1 (16)	8.2 ± 2.6 (12)
	Posttrtmt	2.9 ± 0.8 (16)	11.1 ± 3.7 (12)
	Termination	4.37 ± 1.4 (16)	3.4 ± 1.1 (12)
Corticosterone (ng/ml)	Pre-exp	67.0 ± 7.0 (16)	82.1 ± 12.4 (12)
	Pretrtmt	151.2 ± 14.2 (15)	115.9 ± 15.0 (12)
	Posttrtmt	81.2 ± 16.9 (16)	88.4 ± 21.5 (12)
	Termination	187.8 ± 24.3 (15)	208.3 ± 48.5 (12)

Means based on average values for each rank category per cage (see Methods). Sample sizes in parentheses.

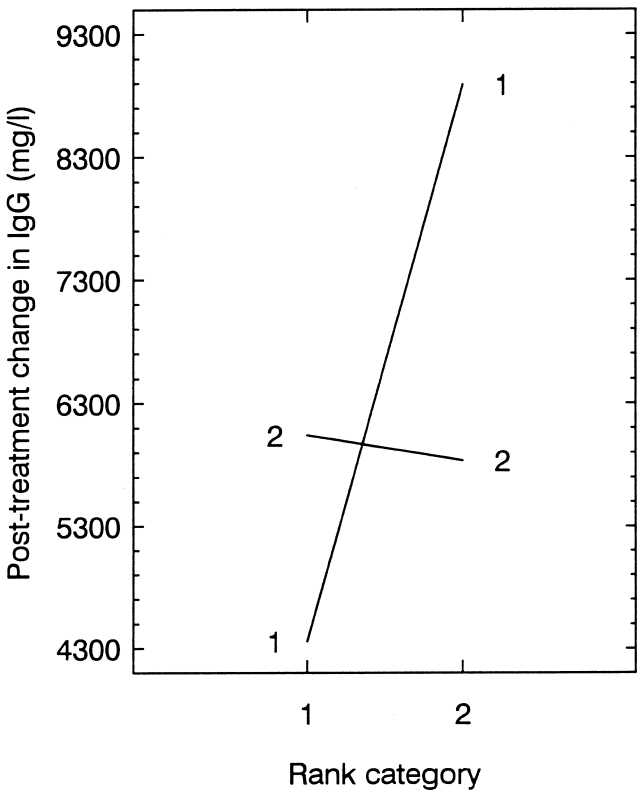

FIG. 3. The interaction between rank category and treatment from ANOVA of posttreatment total IgG concentration. Least-squares deviation bars omitted from mean values for clarity. Treatments as in Fig. 1. See text.

haemagglutination titre, and the change in total IgG concentration and body weight over the same period as independent variables. No significant relationships emerged when treatments were combined but, when analyzed separately, there was a strong positive relationship with thymus weight among ATS-treated mice [$t(14) = 2.43$, $p < 0.005$, Fig. 5], showing that the smaller the relative weight of the thymus gland, the greater the reduction in testosterone concentration. When thymus weight had been taken into account, however, there was a significant *negative* relationship with the change in IgG concentration [$t(14) = -3.50$, $p < 0.004$].

No significant effects of treatment or rank category emerged for corticosterone concentration and there were no significant correlations between change in corticosterone and testosterone concentrations over any phase of the experiment.

Analysis of changes in serum hormone concentration during pre- and posttreatment periods of grouping accorded with results from previous experiments with groups of male CFLP mice [e.g., (5)], with the exception of posttreatment ATS groups. In all cases except for posttreatment ATS mice, testosterone concentration tended to decline over the period of grouping, and in all cases corticosterone concentration tended to increase (Table 2). There were no significant differences in the degree of change between treatments for either hormone.

Effect of Treatment on Body and Organ Weights

Prior to separation and treatment, there was no significant difference in body weight between mice subsequently given different treatments; neither was there a difference between rank categories. However, ANOVA of the change in body weight over the period of isolation and treatment revealed a significant difference between ATS- and NRS-treated animals, with the former showing an increase in weight compared to a decrease in NRS controls [$F(1, 24) = 12.34$, $p < 0.01$]. As a result, immediate posttreatment weight differed significantly between the 2 groups [$F(1, 24) = 5.48$, $p < 0.03$]. However, after the second period of grouping, body weight (terminal) again showed no difference between treatment groups. There were no significant main or interaction effects of rank.

Two-way ANOVA of organ weights (as % body weight) other than the thymus gland (see above) revealed a significant treatment effect only in case of the spleen, which was significantly larger in ATS-treated animals [$F(1, 25) = 14.27$, $p < 0.001$].

Effect of Treatment on Behaviour

Previous work (3–6) has indicated a role of aggressiveness in status-related immunodepression and reduced resistance to disease among male CFLP mice. We, therefore, expected treatment with ATS to result in a reduction in the amount of aggression initiated. Because there was no interaction between treatment and rank category in immunocompetence measures (see above), however, there was no reason to expect a differential effect of treatment with respect to rank on aggression. Two-way ANOVA of the difference in the total amount of aggression initiated before and after treatment (post- minus pretreatment number of occurrences, taking post- minus pretreatment change in

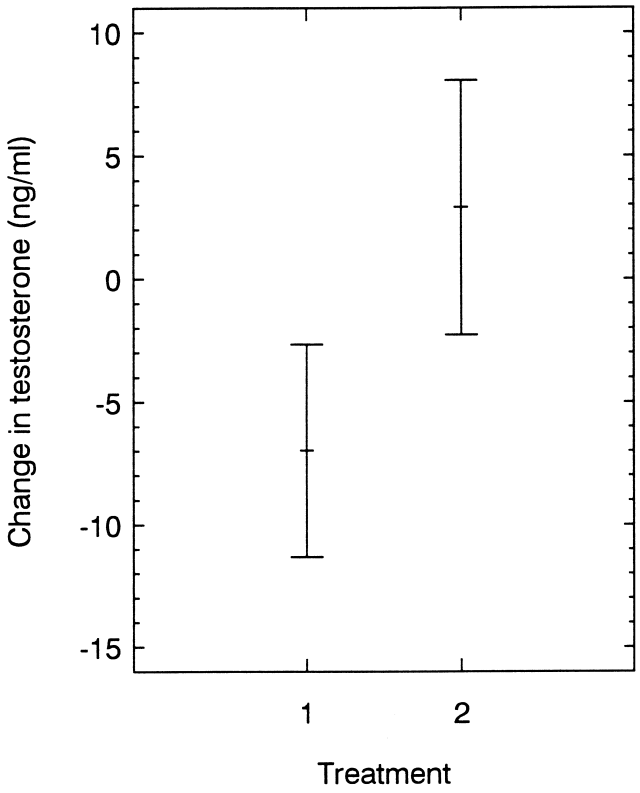

FIG. 4. Least-squares means from ANOVA of the change in testosterone concentration across the 6-day period of treatment in ATS- and NRS-treated mice. Treatments as in Fig. 1. See text.

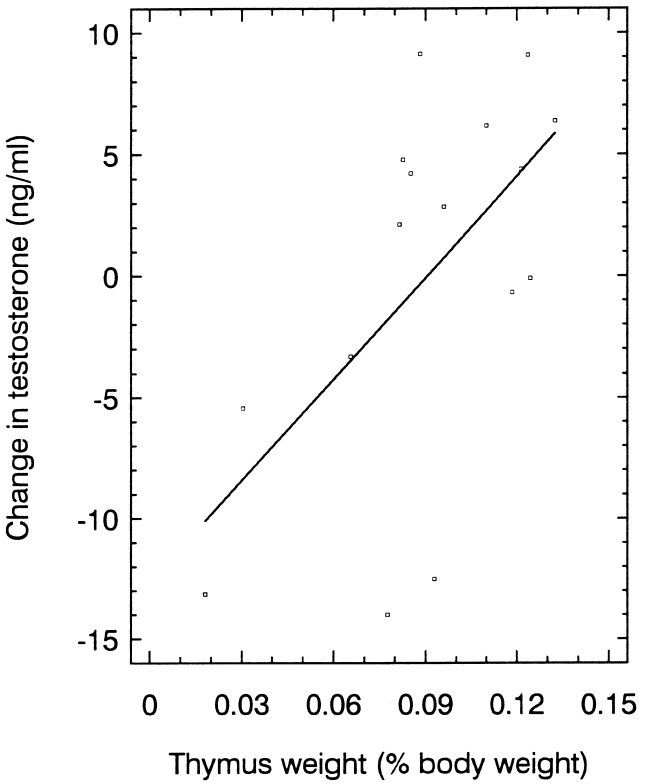

FIG. 5. Component effect from partial regression analysis of the relationship between thymus weight (% terminal body weight) and the change in testosterone concentration among ATS-treated mice over the 6-day period of treatment. Regression equation: y = −31.81 + 139.41(th) − 0.02(tigg), where *th* = relative thymus weight and *tigg* = post- minus pretreatment change in total IgG concentration. No other independent variable entered the equation. See text.

body weight into account as a covariate, see above) confirmed a treatment effect, with the number of initiations being reduced to a greater extent after ATS treatment than after NRS treatment [$F(1, 23) = 3.04$, 1-tailed $p < 0.05$, Fig. 6]. There was also a significant difference between rank categories, with the decline being much greater among those of high rank than among low ranking animals [$F(1, 23) = 13.45$, $p < 0.005$], but no significant interaction between treatment and rank category. There was no significant effect of change in body weight and no significant pretreatment difference in aggressiveness between the 2 treatment groups. Analysis of the degree of escalation in aggressive encounters revealed a reduction in ATS mice compared with NRS controls (mean ± SE change in ATS mice = −1.03 ± 0.41; in NRS mice = 0.07 ± 0.82), but the difference was not significant. Contrary to expectation, there was no corresponding increase in social investigation, and no significant change in other social behaviours (allogrooming, mounting) sometimes associated with aggression (20,24).

Compromising immune function might also be expected to depress general activity if metabolic demand is likely further to depress immunocompetence. In keeping with this, 2-way ANOVA showed a significant effect of treatment on the difference in the number of times mice were recorded in general locomotory activity (combined mobile, climb, dig in sawdust, investigate cage, in Table 1, [$F(1, 24) = 6.60$, 1-tailed $p < 0.01$] and sleeping [$F(1, 23) = 5.53$, 1-tailed $p < 0.03$] before and after treatment. Levels of locomotory activity were maintained after treatment among NRS animals, but declined in ATS-treated mice, and the converse was true for time spent sleeping (Fig. 7a,b). Because ATS treatment resulted in a greater decline in PCV [$F(1, 24) = 5.42$, $P < 0.03$], we controlled for potential effects of erythrocyte loss on the tendency to sleep, by taking the change in PCV into account as a covariate in the above analysis. There were no significant covariate, main, or interaction effects relating to rank category. There were also no significant pretreatment differences in locomotory activity or sleeping between the 2 treatment groups. There were no significant changes in any other behaviour category (Table 1). Although both aggression and locomotory activity decreased after ATS treatment, there was no significant correlation between the two [$r(28) = 0.24$, NS], implying that the change in aggression was not simply a trivial consequence of an overall reduction in activity.

Relationships Between Immunocompetence, Hormone Concentration, and Behaviour

If the above effects of treatment on subsequent behaviour reflected modulation of behaviour in response to immunodepression, we should expect a positive association between the degree of change in immunocompetence over the period of treatment and the corresponding degree of change in aggression, and a negative association with the change in sleeping. To test this, we used stepwise partial regression analysis (combining treatments and rank categories) with change in behaviour (post- minus pretreatment number of occurrences) as the dependent variable, and thymus weight, haemagglutination titre, and (post- minus pre-

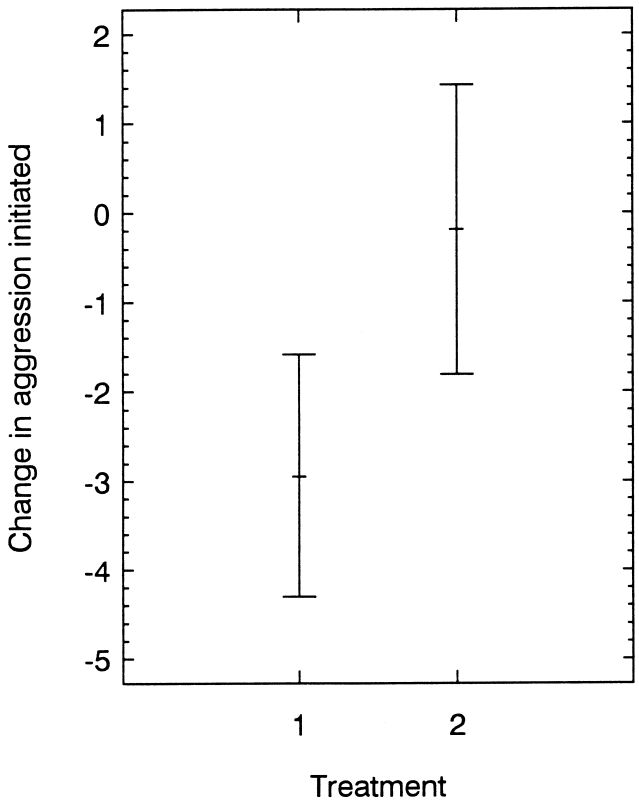

FIG. 6. Least-squares means from ANOVA of the post- minus pretreatment change in the amount of aggressive behaviour initiated by ATS- and NRS-treated mice. Treatments and bars as in Fig. 1.

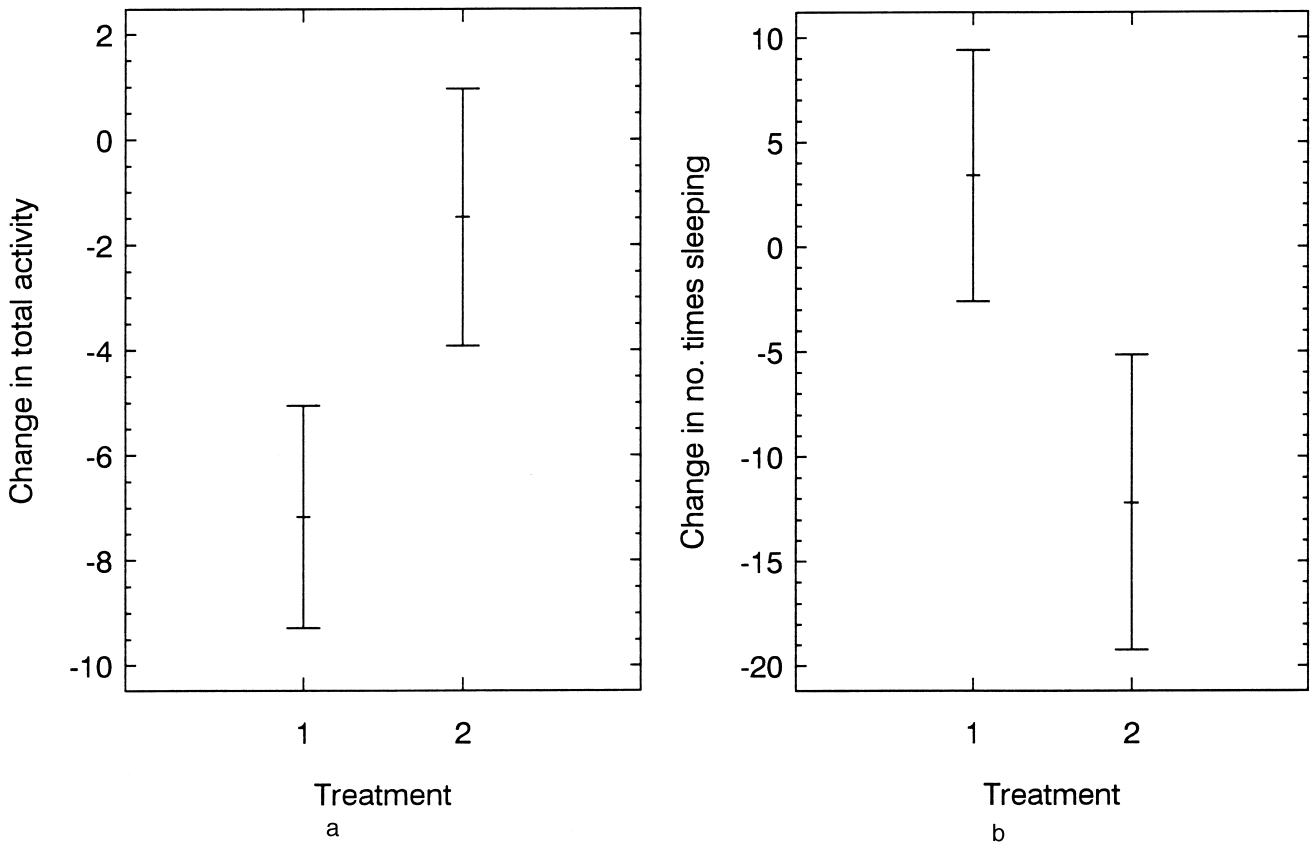

FIG. 7. Least-squares means from ANOVA of the post- minus pretreatment change in the amount of (a) locomotory behaviour and (b) sleep among ATS- and NRS-treated mice. Treatments and bars as in Fig. 1.

treatment) changes in body weight and total IgG and testosterone concentrations as independent variables. Change in corticosterone concentration was not included because no treatment effect emerged for corticosterone (see above) and previous studies of CFLP and other strains of mice [e.g., (9)] suggest that changes in corticosterone concentration in the context of social interactions are consequent on, rather than preludes to, individual differences in aggression [but see (19) for associations in rats]. The results showed that the change in aggression was positively related to the change in total IgG [$t(26) = 3.33$, 1-tailed $p < 0.002$, Fig. 8], but not to thymus weight or haemagglutination titre. Animals showing the greatest reduction in IgG over the period of treatment, thus, showed the greatest subsequent reduction in aggression. The relationship was significant for both ATS- and NRS-treated mice when analyzed separately [ATS, $t(14) = 3.06$, 1-tailed $p < 0.01$; NRS, $t(10) = 3.29$, 1-tailed $p < 0.01$], implying that the treatment effect on aggression above was due to the greater reduction in immunocompetence among ATS-treated mice, rather than to some other effect of ATS treatment.

Despite the absence of a significant difference between treatments for social investigation (see above), partial regression analysis of the change in investigatory activity after treatment in ATS mice nevertheless revealed a significant positive relationship with thymus weight [$t(14) = 3.58$, 1-tailed $p < 0.01$, Fig. 9]. Investigation thus increased to a greater extent in those ATS-treated animals maintaining a greater relative thymus weight. There were no significant relationships among NRS mice or when treatments were combined.

Likewise, regression analysis of NRS and combined treatments failed to yield any significant relationships for locomotory activity or sleep, but strongly significant associations with thymus weight emerged among ATS-treated animals (for locomotory activity, $t(14) = 4.77$, 1-tailed $p < 0.0001$, Fig. 10a; for sleep, $t(14) = -3.78$, 1-tailed $p < 0.002$, Fig. 10b]. ATS-treated mice, thus, reduced the amount of locomotory activity but maintained the amount of sleep after treatment in proportion to the reduction in relative thymus weight. No other significant relationships emerged for sleep.

If aggression was damped in response to a preceding decline in immunocompetence, was there any evidence that greater levels of posttreatment aggression, either initiated or received (6) correspondingly damped the apparent posttreatment recovery (at least as measurable by the change in total IgG)? Stepwise partial regression analysis with the change in IgG across the posttreatment period of grouping as the dependent variable, and posttreatment aggression, social investigation, sleep, and change in body weight and testosterone concentration as independent variables, failed to reveal any effect of aggression. However, posttreatment sleep showed a significant *negative* association with posttreatment change in IgG [$t(26) = -2.11$, $p < 0.05$]. When treatments were analyzed separately, the trend remained only among ATS-treated mice [$t(14) = -2.91$, $p < 0.02$) which, in a further analysis, also showed a significant negative relationship between posttreatment sleep and thymus weight [$t(14) = -3.29$, $p < 0.006$]. No significant relationships were found for social investigation.

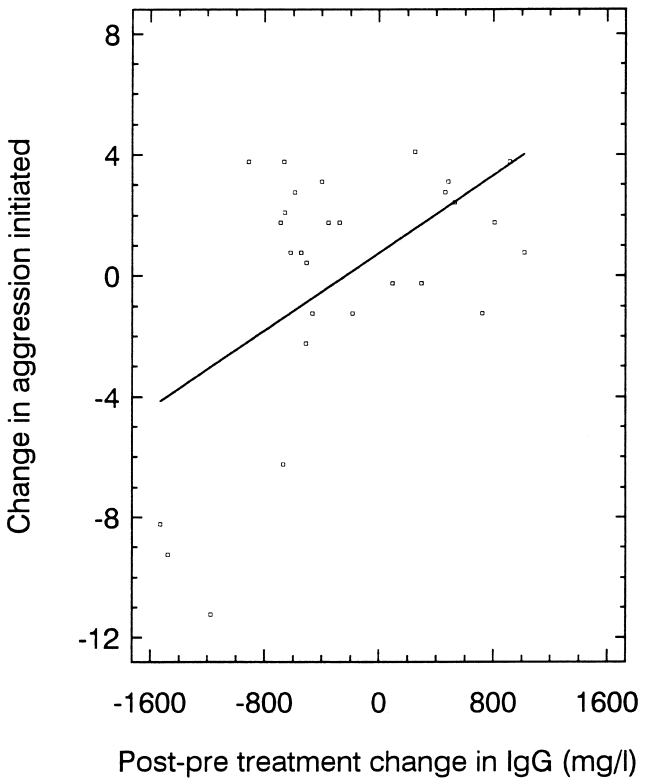

FIG. 8. Component effect from partial regression analysis of the relationship between the change in total IgG concentration over the 6-day period of treatment and the post- minus pretreatment change in the amount of aggression initiated (treatments combined). Regression equation: $y = -0.10 + 0.003(\text{tigg})$. Abbreviations as in Fig. 5. No other independent variables entered the equation. See text.

DISCUSSION

Our expectation that induced immunodepression would result in downregulation of potentially immunodepressive hormones and aggressive behaviour was borne out by the results. However, apart from an interaction in posttreatment recovery of IgG concentration, there was little evidence for a difference between rank categories in response to treatment.

The absence of a detectable antibody response to the injection of SRBC confirmed that ATS-treated mice were severely immunodepressed, and the reduction in total IgG concentration following treatment indicated that pre-existing immune responses were also temporarily affected. Total IgG concentration declined over the period during which mice were separated and treated with ATS, but then increased as the immunodepressive effect waned following the cessation of treatment and mice presumably responded to the foreign proteins in the rabbit serum. Treatment with ATS during the experiment was associated with a reduction in relative thymus weight and an increase in spleen weight, the latter an expected consequence (12) of increased haemopoiesis following partial reduction in erythrocytes as a consequence of some cross-reactivity of ATS. The PCV fell marginally in ATS-treated mice, but values remained well within the limit tolerated by mice.

The change in IgG was reflected by a simultaneous change in serum testosterone concentration. However, when relationships were partialled out, the decline in testosterone among ATS-treated mice correlated with a reduction in relative thymus weight, rather than IgG concentration, supporting the idea of some thymus-mediated regulation of hormone secretion (15). That testosterone, but not corticosterone, tracked changes in immunocompetence measures is consistent with previously reported differences in the apparent modulation of the two hormones in relation to current immune status (5,32).

Our interpretation of the association between testosterone and immunocompetence in terms of downregulating a potential immunodepressant when immune function is impaired is supported by the observed behavioural changes. ATS-treated mice showed a reduction in the amount of aggression initiated and general activity relative to pretreatment levels when groups were reconstituted after treatment, and levels of activity, particularly aggression, were expected to be high (3,29), but maintained pretreatment levels of sleep. The fact that the reduction in aggression correlated with that in total IgG concentration across the period of treatment, in both ATS and naive serum control mice, strengthens the conclusion that the treatment effect was the result of increased immunodepression among ATS mice. The relationships between immunocompetence and time spent in social investigation and sleep were confined to ATS-treated animals and related to the reduction in relative thymus weight, rather than IgG concentration. As in the case of aggression, however, there was no association with testosterone concentration. The lack of any independent association between testosterone concentration and behaviour concurs with previous findings, in which evidence for relationships between testosterone levels and aggression among male CFLP mice is weak [(5,32) see also (10), but see

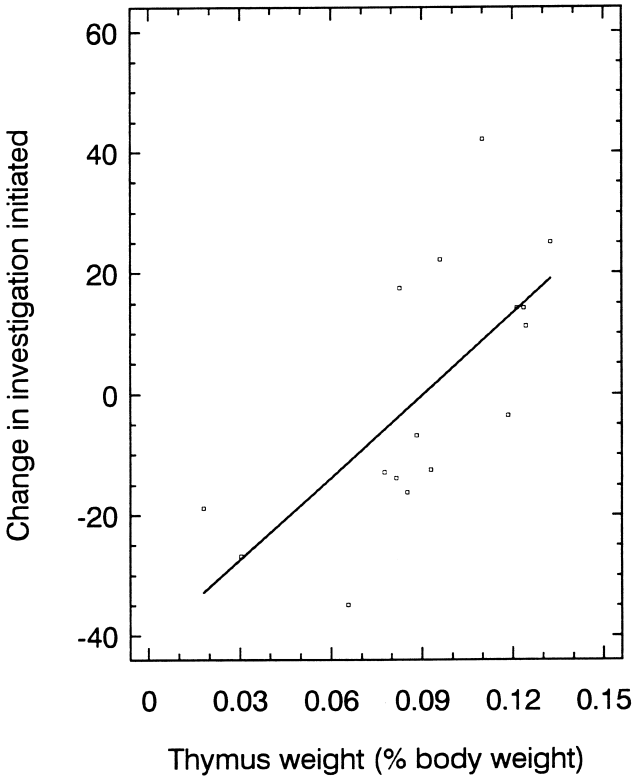

FIG. 9. Component effect from partial regression analysis of the relationship between thymus weight (% terminal body weight) and the post-minus pretreatment change in the amount of social investigation initiated by ATS-treated mice. Regression equation: $y = -43.24 + 453.81(\text{th})$. Abbreviations as in Fig. 5. No other independent variables entered the equation. See text.

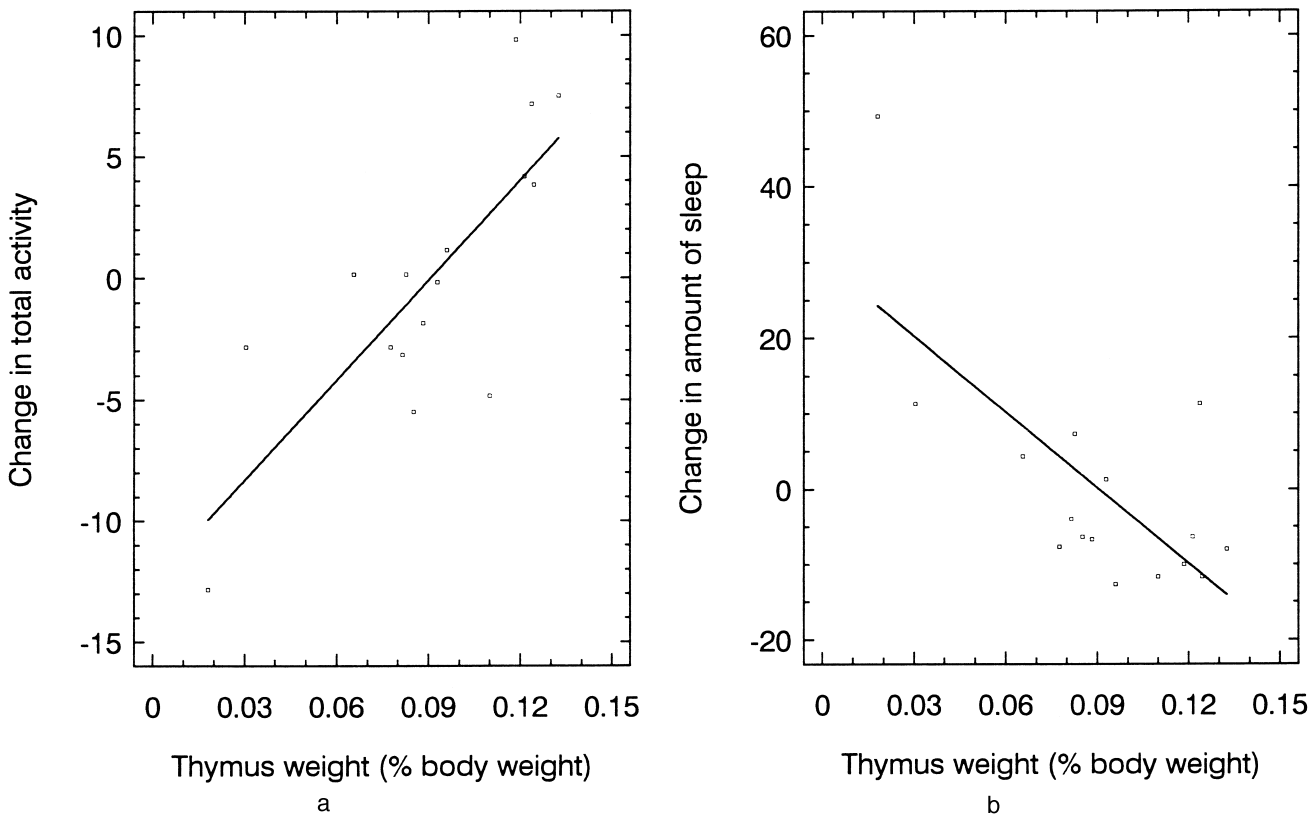

FIG. 10. Component effect from partial regression analysis of the relationship between thymus weight (% terminal body weight) and post- minus pretreatment change in the amount of (a) locomotor behaviour and (b) sleep among ATS-treated mice. Regression equations: (a) $y = -19.59 + 137.15(th)$, (b) $y = 32.05 - 335.53(th)$, respectively. Abbreviations as in Fig. 5. No other independent variables entered the equations. See text.

(4)], and suggests that hormonal and behavioural changes are independent responses to immunodepression.

Surprisingly, the amount of aggression initiated or received did not affect the posttreatment rise in IgG or any of our other measures of immunocompetence. However, the smaller increase in IgG among ATS-treated high-rank animals suggested an indirect association between aggressiveness and recovery of antibody response. Although posttreatment aggression had little impact on changes in immunocompetence measures, there was an, at first sight, counterintuitive negative relationship between time spent sleeping and both thymus weight and rise in IgG (implying that animals that slept more ended up with smaller thymus glands and showed less recovery in IgG concentration). Because the increase in sleep after treatment was a function of reduced thymus weight, however, a plausible explanation for the posttreatment relationship might be that more severely immunocompromised individuals both slept more and showed a weaker recovery of thymus cells and IgG levels, but that behaviour and immunocompetence measures were not causally related.

Overall, the results of the experiment strongly support the idea that aspects of behaviour and physiology affecting immunocompetence may be modulated in relation to current immune status. They complement previous results relating to spontaneous changes in hormone concentrations, behaviour, and immune function in male CFLP mice in suggesting an important role of immune function in behavioural and physiological decision-making.

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REFERENCES

1. Alexander, J.; Stimson, W. H. Sex hormones and the course of parasitic infection. Parasitol. Today 4:198–193; 1988.
2. Barnard, C. J.; Hurst, J. L. Welfare by design: the natural selection of welfare criteria. Anim. Welfare (in press).
3. Barnard, C. J.; Behnke, J. M.; Sewell, J. Social behaviour, stress and susceptibility to infection in house mice (*Mus musculus*): effects of duration of grouping and aggressive behaviour prior to infection on susceptibility to *Babesia microti*. Parasitology 107:183–192; 1993.
4. Barnard, C. J.; Behnke, J. M.; Sewell, J. Social behaviour and susceptibility to infection in house mice (*Mus musculus*): effects of group size, aggressive behaviour and status-related hormonal responses prior to infection on resistance to *Babesia microti*. Parasitology 108:487–496; 1994.

5. Barnard, C. J.; Behnke, J. M.; Sewell, J. Social status and resistance to disease in house mice (*Mus musculus*): status-related modulation of hormonal responses in relation to immunity costs in different social and physical environments. Ethology 102:63–84; 1996.
6. Barnard, C. J.; Behnke, J. M.; Sewell, J. Environmental enrichment, immunocompetence and resistance to *Babesia microti* in male laboratory mice. Physiol. Behav. 60:1223–1231; 1996.
7. Beden, S. N.; Brain, P. F. Effects of treatment with sex steroids on the primary immune response to sheep red blood cells in castrated mice. IRCS Med. Sci. 12:638; 1986.
8. Behnke, J. M.; Parish, H. A. Transfer of immunity to *Nematospiroides dubius*: co-operation between lymphoid cells and antibodies in mediating worm expulsion. Paras. Immunol. 3:249–259; 1981.
9. Brain, P. F. Social stress in laboratory mouse colonies. In: UFAW, ed. Laboratory animal welfare research: rodents. Potters Bar: UFAW; 1988:49–61.
10. Creel, S.; Wildt, D.; Monfort, S. L. Aggression, reproduction and androgens in wild dwarf mongooses: a test of the challenge hypothesis. Am. Nat. 141:816–825; 1993.
11. Dace, J. V.; Lewis, S. M. *Practical haematology*. 8th ed. Churchill Edinburgh: Livingstone; 1995.
12. Doenhoff, M. J.; Leuchars, E. Effects of irradiation, anti-thymocyte serum and corticosteroids on PHA and LPS responsive cells of the mouse. Int. Arch. Allergy Appl. Immunol. 53:505–514; 1977.
13. Dracott, B. N.; Smith, C. E. T. Hydrocortisone and antibody response in mice. I. Correlations between serum cortisol levels and cell numbers in thymus, spleen, marrow and lymph nodes. Immunology 38:429–435; 1979.
14. Folstad, I.; Karter, A. J. Parasites, bright males and the immunocompetence handicap. Am. Nat. 139:603–622; 1992.
15. Grossman, C. J. Interactions between the gonadal steroids and the immune system. Science 227:257–261; 1985.
16. Grossman, C. J.; Roselle, G. A. The control of immune response by endocrine factors and the clinical significance of such regulation. Progr. Clin. Biochem. Med. 4:9–56; 1986.
17. Hurst, J. L. The priming effects of urine substrate marks on interactions between male house mice, *Mus musculus domesticus* Shwarz and Schwarz. Anim. Behav. 45:55–81; 1993.
18. Hurst, J. L.; Fang, J.; Barnard, C. J. The role of substrate odours in maintaining social tolerance between male house mice, *Mus musculus domesticus*: relatedness, incidental kinship effects and the establishment of social status. Anim. Behav. 48:157–167; 1994.
19. Hurst, J. L.; Barnard, C. J.; Hare, R.; Wheeldon, E. B.; West, C. D. Housing and welfare in laboratory rats: status-dependent time-budgeting and pathophysiology in single sex groups maintained in open rooms. Anim. Behav. 52:335–360; 1996.
20. Kareem, A. M.; Barnard, C. J. The importance of kinship and familiarity in social interactions between mice. Anim. Behav. 30:594–601; 1982.
21. Kotani, M.; Korenaga, M.; Nawa, Y.; Kotani, M. Inhibition by testosterone of immune reactivity and of lymphoid regeneration in irradiated and marrow reconstituted mice. Experientia 34:1343–1345; 1974.
22. Lance, E. M.; Medawar, P. B.; Taub, R. N. Antilymphocyte serum. Adv. Immunol. 17:2–92; 1973.
23. Levey, R. H.; Medawar, P. B. Some experiments on the action of antilymphoid sera. Ann. NY Acad. Sci. 129:164–177; 1966.
24. Mackintosh, J. H. Behaviour of the house mouse. Symp. Zool. Soc. Lond. 47:337–365; 1981.
25. Mancini, G.; Carbonara, A. O.; Heremans, J. F. Immunochemical quantitation of antigens by single radial immundiffusion. Immunochem. 2:235–254; 1965.
26. Mooradian, A. D.; Morley, J. E.; Korenman, S. G. Biological actions of androgens. Endocrine Rev. 8:1–28; 1987.
27. Owens, I. P. F.; Short, R. V. Hormonal basis of sexual dimorphism in birds: implications for new theories of sexual selection. Trends Ecol. Evol. 10:44–47; 1995.
28. Peng, X.; Lang, C. M.; Drodzdowicz, C. K.; Ohlsson-Wilhelm, B. K. Effect of cage population density on plasma corticosterone and peripheral lympocyte populations of laboratory mice. Lab. Animals 23:302–306; 1989.
29. Poole, T. B.; Morgan, H. D. R. Differences in aggressive behaviour between male mice (*Mus musculus* L.) in colonies of different sizes. Anim. Behav. 21:788–795; 1973.
30. Rife, S. U.; Marquez, M. G.; Escalante, A.; Velich, T. The effect of testosterone on the immune response. I. Mechanism of action on antibody-forming cells. Immunol. Inv. 19:259–270; 1990.
31. Roberts, C. W.; Satoskar, A.; Alexander, J. Sex steroids, pregnancy-associated hormones and immunity to parasitic infection. Parasitol. Today 12:382–388; 1996.
32. Smith, F. V.; Barnard, C. J.; Behnke, J. M. Social odours, hormone modulation and resistance to disease in male laboratory mice (*Mus musculus*). Anim. Behav. 52:141–153; 1996.
33. Taub, R. N. The biological effects of heterologous antilymphocyte serum. Progr. Allergy 14:208–258; 1970.
34. Turk, J. L.; Willoughby, D. A. Central and peripheral effects of antilymphocyte sera. Lancet 1:249–251; 1974.
35. Wedekind, C.; Folstad, I. Adaptive or nonadaptive immunosuppression by sex hormones? Am. Nat. 143:936–938; 1994.
36. Wingfield, J. C.; Hegner, R. E.; Dufty, A. M. Jr.; Ball, G. F. The ''challenge hypothesis:'' theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. Am. Nat. 136:829–846; 1990.