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Assessment of stress in non-human primates: application of the neutrophil activation test

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Abstract

A technique measuring leukocyte (neutrophil) activity was used to examine differences between stress levels in a breeding colony of rhesus macaques housed in either a traditional caging system or open-rooms. The leukocyte activation test measured the degree to which blood from the two treatment groups could launch a further neutrophil response (superoxide production) to an in vitro *challenge. Animals housed in a traditional caging system produced a significantly lower leukocyte response than animals housed in open-rooms, indicating that there was a higher level of stress associated with caged housing than open-room housing. This was not influenced by whether animals were physically restrained or trained to stand for a sedating injection. No differences were found between treatment groups in leukocyte numbers or composition. This study validates the use of the leukocyte activation test to assess physiological stress levels in non-human primates and demonstrates the animal welfare benefits of open-room housing over traditional laboratory caging systems.*

Keywords: *animal welfare, housing refinement, leukocyte activity,* Macaca mulatta*, rhesus macaques, stress*

Introduction

Caging and stress

A considerable body of literature exists on the influence of housing conditions on the physiological measures of primate stress (eg Crockett *et al* 2000; Mendoza *et al* 2000; Honess & Marin 2005). Research demonstrates that stress levels cause changes that affect a number of biological functions, including immune competence (Moberg 1985; Raberg *et al* 1998; Maule & VanderKooi 1999), brain structure and function (Uno *et al* 1989; Sapolsky 1996, 2003), and reproduction (Carlstead & Shepherdson 1994; Pottinger 1999; Ha *et al* 2000).

There is a clear effect of overall cage size (eg Draper & Bernstein 1963; Paulk *et al* 1977), and its structural complexity (eg Reinhardt 1996; Röder & Timmermans 2002; Buchanan-Smith *et al* 2004; Wolfensohn & Honess 2005), on the psychological well-being of primates. An increase in space and its enrichment allows increases in socialisation and creates opportunities for training animals to cooperate with husbandry and veterinary procedures (Wolfensohn 2004). This has clear implications for the maintenance of captive animals for research and for breeding.

Measurement of stress

Traditional measurements of physiological stress have focused on the detection of cortisol levels in the blood plasma, faeces, urine or saliva (Mendoza *et al* 2000; Theorell 2003), or on monitoring autonomic responses, such as changes in heart rate or blood pressure (Porges 1985; Line *et al* 1989a,b). The implantation of telemetry devices to measure cardiovascular parameters may compound and confound stress measures, and less invasive methods of monitoring are ethically more acceptable. However, the collection of faeces, urine or saliva samples for cortisol assay is practically complex, particularly from group-housed animals on a forage substrate. Furthermore, the interpretation of cortisol assay results is complicated by considerable individual variation (Montane *et al* 2002), a natural circadian variation in cortisol levels (Sousa & Ziegler 1998; Mendoza *et al* 2000; Theorell 2003), the fact that a cortisol response is associated with some non-stress stimuli, and that some stress responses may not involve elevated cortisol levels (Moberg 2000). Although low concentrations of cortisol are typically associated with low stress, chronically stressed primates are known to exhibit hypocorticolism (Mendoza *et al* 2000). These problems have contributed to an increasing dissatisfaction with the use of cortisol to measure stress levels (Crockett *et al* 2000; Moberg 2000).

This study monitors the activity of leukocytes, which have been shown to increase oxygen uptake in response to bacterial challenge in order to produce oxygen free radicals that kill bacteria (Hu *et al* 1999; Halliwell & Gutteridge 2000). Psychological stress produces the same response (Ellard *et al* 2001) and can also influence the number and distribution of leucocytes, and the expression of their

Table 1 Analysis of leukocyte activity (relative light units [RLUs]) in rhesus macaques kept in a traditional caging system versus open-room housing conditions. Figures in brackets are one standard error, ns = non significant, ** = $P < 0.01$.

	Leukocyte activity			Leukocyte numbers and composition			
	PMA 10^{-3} mol l^{-1}	PMA 10^{-5} mol l^{-1}	Unchallenged	Leukocytes 10^{9} l^{-1}	% Neutrophils	% Lymphocytes	Neutrophil-lymphocyte ratio
Caged (n = 10)	7960.68 (1178.46)	2069.49 (400.33)	1923.25 (402.11)	9.76 (1.32)	58.30 (6.68)	40.50 (6.51)	2.48 (0.77)
Open-room (n = 43)	16157.11 (2840.28)	3258.02 (525.09)	1605.92 (150.86)	9.38 (0.55)	62.47 (2.43)	36.37 (2.40)	2.28 (0.24)
Mann-Whitney *U*	97.00	184.00	190.00	215.00	186.50	185.00	185.00
P value (2-tailed)	0.007**	0.481 ns	0.570 ns	1.000 ns	0.517 ns	0.495 ns	0.495 ns

Table 2 Analysis of the effect of capture technique (standing for injection versus being netted) on leukocyte activity (RLUs) in rhesus macaques housed in open-rooms. Figures in brackets are one standard error, ns = non significant.

	PMA 10^{-3} mol l^{-1}	PMA 10^{-5} mol l^{-1}	Unchallenged
Standing (n = 13)	24129.67 (8933.49)	3473.01 (773.25)	1759.42 (301.11)
Netted (n = 22)	13766.96 (1219.71)	3856.59 (884.81)	1668.72 (222.37)
Mann-Whitney *U*	136.0	134.0	131.0
P value (2-tailed)	0.811 ns	0.759 ns	0.682 ns

adhesion receptors (Dhabhar *et al* 1995; Kang *et al* 1996; Goebel & Mills 2000). While changes in cell numbers and adhesion molecules are not exclusively associated with altered cellular activity and function (Mian & Marshall 1993), an extensive range of physical and psychological stressors do produce a rapid response in leucocytes that can affect the immune system's ability to cope with an ongoing or potential challenge (Dhabhar *et al* 1995).

This technique has already been demonstrated as an appropriate measurement of psychological stress in humans (Ellard *et al* 2001; Mian *et al* 2003) and in wild badgers in the field (McLaren *et al* 2003; Montes *et al* 2004). This study aims to examine its use in non-human primates by investigating the stress levels of rhesus macaques (*Macaca mulatta*) housed in different conditions (a traditional caging system versus open-rooms) and the extent to which any difference in stress levels is affected by capture method (physical restraint versus trained standing for injection).

Materials and methods

Study subjects

The animals used in this study were part of the Oxford University rhesus macaque breeding colony housed at Harlan, UK. This specific-pathogen-free colony consisted of approximately 200 monkeys including 16 single male breeding groups of 4–11 females and their unweaned infants, which were transferred to peer groups at 12–18 months. All macaques were group-housed indoors in either a traditional caging system of three linked modules of typical reinforced stainless steel two-tier laboratory cages (each module: 1.20 × 1.20 × 2.0 m, width × depth × height; total: 2.88 × 3 = 8.64 m^3), or in open-rooms (approximate total average of 22.8 m^3 per group) containing either these cages with the fronts removed or no caging at all. These different housing conditions resulted from a progressive move toward all open-room housing as part of a programme of refinement of the colony's husbandry and management (Wolfensohn 2004).

All housing conditions had a woodchip forage substrate spread on the floor. Each room contained a flexible arrangement of wooden furniture, wooden shelves and hanging items (eg tyres), had ceramic tiles or forex sheeting (Lonza Ltd, Basle, Switzerland) on the walls, protected light and electrical fittings, and a secondary door. Waste solids were removed every two days and the substrate changed weekly; rooms were thoroughly washed every 10–14 days, and cages were thoroughly washed every 7–10 days. The animals were fed three times per day: in the morning with monkey chow pellets (Harlan Teklad, Madison, Wisconsin, USA); around noon with foraging mixture (as of Davys 1995); and mid-afternoon with whole, fresh fruit. Water was available *ad libitum*. The animals were maintained under a 12:12 h light:dark lighting regime at a constant temperature (15–24°C) and humidity (45–65%), in accordance with the UK Home Office *Code of Practice for the Housing and Care of Animals in Designated Breeding and Supplying Establishments* (Home Office 1995).

Capture, sedation and blood sampling

This study used 53 adult rhesus macaques aged between 8 and 20 years: 10 from traditional cages (3 males, 7 females) and 43 from open-rooms (6 males, 37 females). These animals had been primarily raised in breeding units, having been weaned at 6–12 months and group-housed for more than 5 years. Those housed in traditional cages were sedated by injection following physical restraint using a squeeze-back mechanism; those in open-rooms were either trained to stand and present their hind-quarters for injection when a technician entered the room (Wolfensohn 2004), or were caught and physically restrained using a net before being injected.

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Sedation was via an intramuscular injection of ketamine hydrochloride at a concentration of 100 mg ml^{-1} , and at a dose of 10 mg kg^{-1} (Ketaset, Fort Dodge, USA). Blood samples were obtained during routine health screening carried out under the Veterinary Surgeons Act 1966 (http://www.rcvs.org.uk/Templates/Internal.asp?NodeID=89679, accessed 4 October 2005) by needle venepuncture of the femoral vein into an EDTA Vacutainer (BD Vacutainer Systems, Plymouth, UK). 30 µl of this blood was taken immediately for the leukocyte assay; the remainder was used for measuring haematology parameters (Celltac MEK–5108K: Kohden, Japan) and differential cell counts from alcohol-fixed blood smears using May-Grunwald and Giemsa stains.

Measurement of leukocyte activity

As described by McLaren *et al* (2003), luminol (5–amino–2, 3–dihydrophthalzine [Sigma A8511]: Sigma-Aldrich Company Ltd, Poole, Dorset, UK) fluoresces in the presence of oxygen free radicals and is therefore used to measure blood chemiluminescence levels. The method used in this study is based on the studies by Mian *et al* (2003) and McLaren *et al* (2003). Appropriate concentrations of luminol (10^{-4} mol l^{-1}) and the microbial product PMA (phorbol 12–myristate 13–acetate [10^{-3} mol l^{-1} and 10^{-5} mol l^{-1}] [Sigma P8139]: Sigma-Aldrich Company Ltd, Poole, Dorset, UK) were used to measure luminescence (relative light units [RLUs]) in a portable chemiluminometer (Junior LB 9509: EG & G Berthold, Germany) for 30 s, every 5 min, over a 45 min period. Three tubes were prepared for each animal's sample: an unchallenged sample (10 µl of blood and 90 µl of luminol), and two with 10 µl of blood, 90 µl of luminol and 10 µl of PMA at each of the two concentrations (high concentration [HC] = 10^{-3} mol l^{-1} and low concentration [LC] = 10^{-5} mol l^{-1}). Tubes were agitated to mix the contents; when not in the luminometer, tubes were maintained at 37°C in a water bath. Blood was challenged with two concentrations of PMA to control for any differential response to a weak or strong concentration. An unchallenged sample allowed assessment of background leukocyte activity.

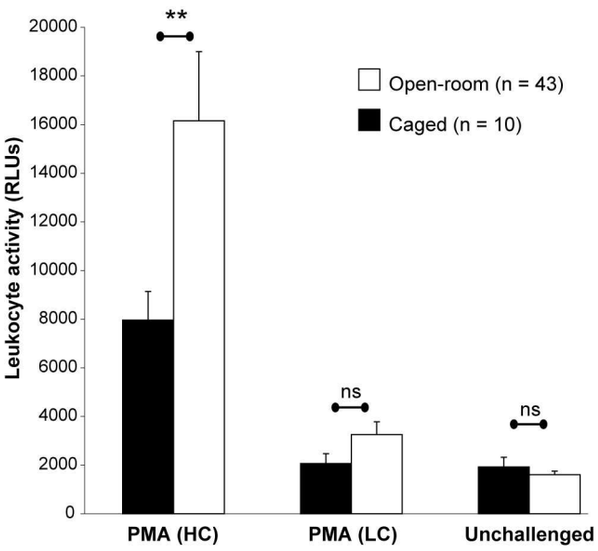
Statistical analyses

Leukocyte activity data were derived from calculating the area under the response curve and dividing by the number of neutrophils, obtained from the differential cell count, to produce a response value that reflected the total response per neutrophil. Data from the two groups (a traditional caging system versus open-rooms) were compared using the non-parametric Mann-Whitney *U* test, using SPSS 11.5® for Windows®.

Results

Comparison of the leukocyte activity of differently housed rhesus macaques revealed that macaques housed in a traditional caging system produced a significantly lower response than those housed in open-rooms to challenge with PMA at high concentration (Mann-Whitney $U = 97.0$, $P = 0.007$) (see Table 1 and Figure 1). A lower response was also found for caged monkeys with the low concentration of PMA, but this was not significant ($U = 184.0$, $P = 0.481$). There was no significant difference in background, unchallenged activity ($U = 190.0$, $P = 0.570$) (see Table 1), and no significant differences were found between the two housing types in the number or composition of leukocytes (see Table 1).

Figure 1



Leukocyte activity in blood challenged with two different concentrations of PMA (HC: 10^{-3} mol l^{-1}; LC: 10^{-5} mol l^{-1}) in rhesus macaques housed in a traditional caging system versus open-rooms. Bars represent the mean total leukocyte activity (+ standard error of the mean) over 45 min, divided by the number of neutrophils (10^{9} l^{-1}); ns = non significant, ** = $P < 0.01$.

As this result could be confounded by differential response to capture technique, ie standing for injection versus physical restraint (net or squeeze-back mechanism), the data from open-room-housed animals, where the capture method (netting or standing) was known, were tested. No significant difference was observed in the leukocyte response to challenge with PMA (see Table 2).

Conclusions

There is considerable evidence to suggest that stress may reduce the effectiveness of the immune system, therefore increasing the risk of infection or disease (Dhabhar *et al* 1995; Kang *et al* 1996; Raberg *et al* 1998). Research suggests that even short-term stress can produce demonstrable and immediate physiological changes in heart rate, blood pressure and the activation of leukocytes (Ellard *et al* 2001; Mian *et al* 2003); these changes depend on the nature and intensity of the stressor (Willard *et al* 1989; McLaren *et al* 2003). In humans, epidemiological studies support the idea that individuals who are more stressed have a suppressed immune system (eg Graham *et al* 1986); stress has also been linked to disease in farm animals (eg Koolhaas *et al* 1999). Therefore, a reduction of immune reactivity as an indicator of stress is not a novel observation (Murata 1989; Haigh *et al* 1997; Maes *et al* 1998). Indeed

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McLaren *et al* (2003) recently reported that in the absence of other underlying pathology an alteration the leukocyte activity is a good indicator of stress.

The animals used in this study were of an extremely high health status, being part of a specific-pathogen-free colony, where they were subjected to regular health screening and continuous surveillance by trained staff. These screenings indicated that there was no difference in the underlying pathology between the treatment groups. All other parameters for the treatment groups were the same as they were housed under regulated conditions and received the same nutrition. This, coupled with the evidence of accommodation size-related stress (see Caging and stress), and clear differences in the space provision in this study, strongly supports that a reduction in the leukocyte responsiveness is an indicator of differences in stress levels, as supported by the published literature (eg Willard *et al* 1989; McLaren *et al* 2003; Mian *et al* 2003; Montes *et al* 2004; Mian *et al* 2005).

This study shows that the leukocyte activation test is appropriate for the measurement of stress in non-human primates, specifically in rhesus macaques. Lower leukocyte response to *in vitro* PMA challenge in animals housed in a traditional caging system, compared with open-room housed animals, demonstrates that cage housing is associated with higher levels of physiological stress. Results also show that this effect was not affected by whether individuals were physically restrained or trained to stand for a sedative injection. A significant difference in the response of the treatment groups to only one of the two PMA concentrations suggests that the leukocyte population may contain functionally different types of neutrophils, responding to different levels or nature of challenge. Where those types that respond to less stressful stimuli have already been used, a further mild challenge may not result in a significant response. Eliciting a further response may, as in this case, require a more powerful stimulus, eg a higher concentration of PMA. The composition of the neutrophil population by type may vary following an acute stressor or as a result of living in chronically stressful conditions.

Unlike previous studies using this technique (ie McLaren *et al* 2003; Montes *et al* 2004), differences in leukocyte activity between the treatment groups were not accompanied by differences in leukocyte number or composition. This may be attributable to differing demographics of the leucocyte populations in the two treatment groups. Stressed animals may be recruiting more immature leukocytes (ie band neutrophils) to replace those used in the stress response, which are represented in cell counts but are not sufficiently mature to emit superoxide in response to a challenge. Therefore, more and less stressed animals may have the same leukocyte population sizes, but more stressed animals may have a greater proportion of neutrophils that are too immature to respond to a further challenge; therefore the total neutrophil response would be less.

Animal welfare implications

This study provides evidence that open-rooms are less stressful than a traditional caging system for groups of rhesus macaques. This leukocyte activation test can be used to indicate the immune competence of animals, highlighting any requirement for additional health monitoring or modification of management timetables, which may include further potentially stressful events (eg change in group composition, weaning of offspring etc). The combination of additional veterinary attention and deferral of further stressful events will optimise the physical and psychological health of an animal, which may otherwise be compromised by compounding an already heightened stress level. This has implications for the animal's reproductive potential as well as its value as an experimental model, as high stress levels are well known to negatively impact on both these factors.

The leukocyte activation test also allows the measurement of physiological stress using much smaller blood samples than for traditional blood cortisol measures (30 µl versus approximately 2 ml). This small amount can be obtained by capillary sampling from a heel prick on trained animals, avoiding the need for sedation and the possible negative consequences (eg haematoma, phlebitis) associated with needle venepuncture sampling. This technique confirms the refinement of housing in the study colony, allows refinement of the management of the animals, and is conducive to the refinement of the sampling technique, with a decrease in blood sample size to approximately 30 µl.

It should, however, be remembered that this technique may not be practical in some situations, for example, in some zoos and sanctuaries where blood sampling may be acceptable for veterinary/diagnostic purposes but not for research purposes where, in the UK, a Home Office licence would be required.

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