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Basal metabolic rate and the evolution of the adaptive immune system

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Vertebrates have evolved an adaptive immune system in addition to the ancestral innate immune system. It is often assumed that a trade-off between costs and benefits of defence governs the evolution of immunological defence, but the costs and benefits specific to the adaptive immune system are poorly known. We used genetically engineered mice lacking lymphocytes (i.e. mice without adaptive, but with innate, immunity) as a model of the ancestral state in the evolution of the vertebrate immune system. To investigate if the magnitude of adaptive defence is constrained by the energetic costs of producing lymphocytes etc., we compared the basal metabolic rate of normal and lymphocyte-deficient mice. We found that lymphocyte-deficient mice had a higher basal metabolic rate than normal mice with both innate and adaptive immune defence. This suggests that the evolution of the adaptive immune system has not been constrained by energetic costs. Rather, it should have been favoured by the energy savings associated with a combination of innate and adaptive immune defence.

Keywords: cost of resistance; ecological immunology; *μMT*; *TCR-β*

1. INTRODUCTION

The vertebrate immune system consists of two parts, innate and adaptive defence. The recognition mechanisms of the innate immune system have low intra-individual diversity and recognize antigens that are general to a wide range of pathogens, for example lipopolysaccharides in bacterial cell walls. However, the diversity of the recognition mechanisms of the adaptive immune system (i.e. T-cell receptors and immunoglobulins) is dramatically increased through somatic mutation and recombination. As a result, this part of the immune system mounts a response that is highly specific for a particular pathogen. Further, an adaptive immune response results in immunological memory, which provides immunity to previously encountered pathogens (e.g. Janeway & Travers 1996). All multicellular organisms have some kind of innate defence, whereas the adaptive component is of more recent evolutionary origin and only occurs in jawed vertebrates (Du Pasquier 1993). The origin and phylogenetic history of the adaptive immune system has been the focus of intense study (Du Pasquier 1993; Thompson 1995; Agrawal *et al.* 1998). By comparison, the selection pressures moulding the adaptive immune system have received little attention (Read & Allen 2000).

In line with the optimality approach (Parker & Maynard Smith 1990), it is commonly assumed that antagonistic selection pressures govern the evolution of immunological defence. In other words, defence should not only have fitness benefits in the form of resistance against infectious organisms, but also costs, and the magnitude of defence should be subject to optimization by natural selection of the trade-off between benefits and costs (e.g. Behnke *et al.* 1992; Frank 1994; Segel & Bar-Or 1999; Shudo & Iwasa 2001). Recently, the expected fitness costs of having and/or using an immune system have been demonstrated empirically, particularly in some invertebrate systems (Boots & Begon 1993; Kraaijveld & Godfray 1997; Webster & Woolhouse 1998; Moret & Schmid-Hempel 2000), but the physiological basis for these fitness costs is as yet poorly understood (but see Siva-Jothy 2000). When it comes to the adaptive immune system, potential mechanisms mediating fitness costs include immunopathology in the form of chronic tissue damage by autoimmune responses and the extra energetic cost of yet another physiological machinery (Behnke *et al.* 1992; Read *et al.* 1995; Råberg *et al.* 1998).

However, an optimality approach to the evolution of the adaptive immune system must take into consideration that innate immune defence was already operating at the incipience of adaptive defence. Because innate defence should also be costly in terms of energy and immunopathology, the net costs of adaptive defence depend on whether or not it could take over some of the roles of innate defence. It is now widely acknowledged that, in modern vertebrates, the innate and adaptive immune systems have become intimately coadapted, interacting and complementing each other; the innate immune system represents a first line of defence, triggering and guiding the slower but more definitive adaptive immune response (Fearon & Locksley 1996; Brown 2001). It seems probable that the innate immune system, in the absence of adaptive immunity in early vertebrates, played a more complete role as a defence against infectious organisms, and thus that the adaptive immune system, to some extent, has replaced the innate. This view, that the two components of defence are partially interchangeable over evolutionary time, is strongly supported by the finding of negative genetic correlations between innate and adaptive

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defence (Cheng *et al.* 1991), because a negative genetic correlation implies that selection for adaptive immunity would, as a correlated evolutionary response, reduce the magnitude of innate defence (or vice versa) (e.g. Falconer & Mackay 1997). Given that adaptive immunity has, to some extent, replaced innate, what are the net costs of adaptive immunity that constrain its magnitude? We focus on energetic costs and investigate whether the adaptive immune system still carries a net cost of energy. In other words, has the evolution of adaptive immunity, as a correlated response, led to a higher basal metabolic rate (BMR)? A high BMR would represent a selective disadvantage whenever food is limited and thus has the potential to constrain the magnitude of adaptive immunity.

2. MODEL ORGANISM

The adaptive immune system consists primarily of T and B lymphocytes. To investigate how the possession of an adaptive immune system affects BMR, we compared the BMR of normal mice with that of knockout mice lacking either T or B cells, or both. We used one mouse strain with a mutation blocking B-cell development, i.e. a mouse with a disrupted B-cell receptor (μ chain) gene (*μMT*) and one type with a mutation blocking *αβ* T-cell development, i.e. with a disrupted T-cell receptor gene (*TCR-β*). These mouse strains were created by targeted knockout of these particular genes, and the mutations are fully recessive (Kitamura *et al.* 1991; Mombaerts *et al.* 1992). By crossing mice heterozygous for both *μMT* and *TCR-β* (see § 3), we generated four categories of mice: (i) mice homozygous for the *μMT* mutation, which consequently lack B cells, (ii) mice homozygous for the *TCR-β* mutation, which lack T cells, (iii) mice homozygous for both *μMT* and *TCR-β*, which lack both B and T cells, and (iv) mice heterozygous (or homozygous for the wild-type alleles) at both loci, which have a normal immune system. For simplicity, the lymphocyte-deficient phenotypes are henceforth denoted B^-, T^- and TB^-, respectively.

We emphasize that both of these mutations act early in the process of differentiation and proliferation of the B- and T-lymphocyte populations, and this not only results in immature and non-functional lymphocytes, but also severely reduces the number of lymphocytes (Kitamura *et al.* 1991; Mombaerts *et al.* 1992). For example, in the fully developed thymus, cells of the early stages (i.e. cells not yet expressing the *β* chain) account for 5% of the total number of thymocytes (Janeway & Travers 1996). Thus, investment in production of T-lymphocytes should be reduced by at least 95% in *TCR-β* mutant mice. As T and B lymphocytes are the primary components of the adaptive immune system, the T^-, B^- and especially TB^- phenotypes represent drastic reductions in the magnitude of adaptive defence.

These mutations also have pleiotropic effects on innate defence such that lymphocyte-deficient mice compensate for their lack of adaptive defence by a higher activity of innate defence, for example by increasing the numbers of neutrophils and natural killer cells (Bründler *et al.* 1996; Langhorne *et al.* 1995; Smelt *et al.* 2000). Under the assumption that the adaptive and innate immune systems have been negatively genetically correlated during the evolution of vertebrate immune defence (Cheng *et al.* 1991), TB^- mice can thus be said to represent the ancestral state, T^- and B^- mice intermediate states and control mice the modern vertebrate.

3. MATERIAL AND METHODS

(a) *Breeding and housing*

μMT mutant breeding pairs were provided by W. Müller (University of Cologne, Germany) and *TCR-β* mutant breeding pairs were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The mutated *μMT* and *TCR-β* loci were backcrossed onto B10.Q-mice (originating from J. Klein, Tübingen, Germany) for six generations. Mice heterozygous for both *μMT* and *TCR-β* were subsequently intercrossed to generate the four categories of mice (see § 2). Mice were bred and kept in the animal facility of the Medical Inflammation Research Group at Lund University in a climate controlled environment with 12 L : 12 D cycles. Animals were housed in polystyrene cages containing wood shavings and fed standard rodent chow and water *ad libitum*. The drinking water was supplemented with Borgal vet (Hoechts) diluted 100-fold. Only females were used in this experiment. The phenotype of each mouse was determined by flow cytometry, which records the presence or absence of B and T lymphocytes (for details see § 3b). Our breeding protocol resulted in 19 B^-, 10 T^-, 12 TB^- and 13 control mice. BMR (for details see § 3c) was measured when mice were 52–101 days old. The variation in age allowed us to investigate whether costs of the adaptive immune system varied with age. Phenotype categories were not biased with respect to age (ANOVA, $F_{3,50} = 1.99$, $p = 0.13$). All statistical analyses were carried out as general linear models in SYSTAT 9 (SPSS Inc.).

(b) *Flow cytometry*

A drop of blood was collected from a tail vein into heparinized tubes. Erythrocytes were lysed with 0.84% NH_4Cl. After washing in ice cold staining buffer (PBS supplemented with 0.5% BSA and 0.02% NaN_3), the cells were incubated with fluorochrome-labelled antibodies to the TCR (H57-597-FITC) and B-cell specific exon of CD45 (RA3-6B2-CyChrome, Pharmingen, La Jolla, CA, USA). H57–597 were purified from culture supernatant and labelled according to the manufacturer's instructions (Molecular probes, Eugene, OR, USA). After a final wash in staining buffer, the cells were analysed on a FACSort with CELLQUEST software (BDB, Mountan View, CA, USA).

(c) *Respirometry*

We used postabsorbtive resting metabolic rate in thermoneutrality as a measure of BMR. Resting metabolic rate was measured as oxygen consumption in an open circuit respirometer. Mice were fasted overnight, and measurements were taken subsequently for 5 h between 10.00 and 17.00. Each mouse was placed in a respirometer chamber (1.6 l) in a climate cabinet at 32 °C, which is within the thermoneutral zone. The respirometer consists of two blocks, each with two parallel channels with identical set-ups and each with an oxygen analyser, a Servomex 1100 A with the sampling system for dry gas. Thus, four mice could be assayed each day. Oxygen concentration was automatically registered on a Grant Squirrel data logger (model 1202) every minute throughout the measurement sessions. Oxygen consumption (ml O_2 min^{-1}) was calculated according to Hill (1972). The value of oxygen consumption used in the analyses was taken as the single lowest value of running 10-min averages during a measurement session.

Table 1. Results of a general linear model of BMR against phenotype, age, age × phenotype and mass of intestine, liver, heart, kidneys and residue in B^-, T^-, TB^- and control mice aged 52–101 days.
(Non-significant terms were successively removed; p to enter or remove was 0.10. β are partial correlation coefficients. Multiple $R^2 = 0.76$. A model with age and (total) body mass, or body mass alone, as covariates, gave virtually identical results. Abbreviation: MS, mean square.)

variable	d.f.	MS	F	β	p
phenotype	3	0.86	3.15	—	0.034
age	1	1.64	5.96	−0.34	0.019
intestine	1	1.56	5.67	0.24	0.021
liver	1	2.12	7.71	0.28	0.008
residue	1	5.80	21.1	0.82	<0.001
error	46	0.28			

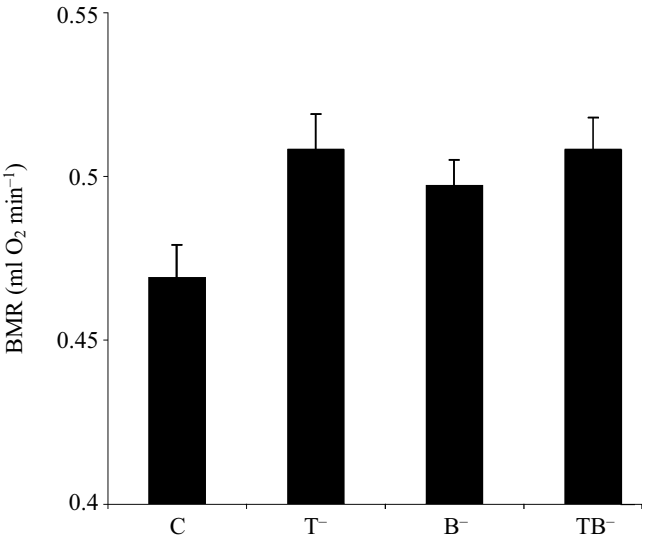

Figure 1. BMR of normal (C, $n = 13$), T^- ($n = 10$), B^- ($n = 19$) and TB^- ($n = 12$) mice. The figure shows least-squares means ± 1 s.e. from a general linear model. There was significant variation among phenotypes in BMR (table 1). Pairwise comparisons revealed that TB^- mice had higher BMR than normal mice (Tukey's test, $p = 0.043$) and that T^- mice tended to have higher BMR than normal mice (Tukey's test, $p = 0.057$).

(d) *Morphometrics*

After BMR measurements were completed, we sacrificed the mice by inhalation of CO_2 and measured the wet mass of the following body components: liver, intestines + stomach (here treated as a unit and henceforth referred to as 'intestines'), heart, kidneys and residue (i.e. total body mass minus the mass of the removed organs). The liver, intestines, heart and kidneys are all recognized for their high mass-specific metabolic rate (e.g. Konarzewski & Diamond 1995). The residue, on the other hand, consists mostly of muscles, adipose tissue etc., which should have relatively low mass-specific metabolic rate. The rationale for weighing these organs was to look for pleiotropic effects of *μMT* and *TCR-β* on body composition, which in turn could affect BMR.

4. RESULTS

In analyses of causes of variation in BMR, it is common to control for variation in body mass. However, since we obtained the mass of different body components, we could investigate the sources of variation in BMR in more detail. We therefore used a general linear model with phenotype as factor and the mass of the different body components as covariates to test for phenotypic differences in BMR. To test if costs of defence varied with age, we also included age in the model, because age could be expected to affect BMR independently of the size of the body components (Blaxter 1989), as well as the phenotype × age interaction. Non-significant terms were successively removed. The final model showed that TB^- mice had *ca.* 10% higher BMR than normal mice (table 1, figure 1).

Presumably, the higher BMR of lymphocyte-deficient mice is attributable to compensatory activation of the innate defence (Bründler *et al.* 1996; Langhorne *et al.* 1995; Smelt *et al.* 2000) to control subclinical infections. However, there is a possibility that the mutations at *μMT* and *TCR-β* had pleiotropic effects on body composition, which in turn inflated (or obscured) the energetic consequences of not having an adaptive immune system. To investigate this, we compared the size of each of the body components between phenotypes by means of ANCOVAs with the body component under consideration as dependent variable, phenotype as factor and age as covariate. In each ANCOVA we also first tested the age × phenotype interaction but excluded it when non-significant. The only evidence for pleiotropic effects as revealed by these analyses was for intestine ($p > 0.10$ for all other main effects and interactions), where there was an interaction between phenotype and age ($F_{3,46} = 3.96$, $p = 0.014$). The interaction was due to young TB^- mice having lower intestine mass than controls, but the mass of their intestine increased faster with age, and from an age of *ca.* 85 days, TB^- mice had higher intestine mass than controls. Because the intestine has a high mass-specific metabolic rate (e.g. Konarzewski & Diamond 1995), the high intestine mass in old TB^- mice could possibly have inflated the phenotypic differences in BMR (even though we controlled for intestine mass in the model above). We therefore repeated the test for phenotypic differences in BMR using a dataset restricted to young (less than or equal to 84 days) TB^- and control mice. In this dataset, there was no interactive effect between age and phenotype on intestine mass ($F_{1,11} = 0.08$, $p = 0.8$), but TB^- had significantly smaller intestines than controls ($F_{1,12} = 11.4$, $p = 0.006$). As in the full dataset, there were no other differences in the size of the different body components (ANCOVAs with phenotype as factor and age as covariate, all $p > 0.10$). A general linear model, constructed in the same way as above, again showed that TB^- mice had significantly higher BMR than controls (TB^-: 0.49 ± 0.012 ml O_2 min^{-1}; controls: 0.43 ± 0.008 ml O_2 min^{-1}; least-squares means ± s.e. from a general linear model; table 2). Thus, there was no indication that the higher BMR of TB^- mice was caused by pleiotropic effects of *μMT* and *TCR-β* on body composition.

5. DISCUSSION

We found that mice lacking B and T lymphocytes had higher BMR than mice with both innate and adaptive

Table 2. Results of a general linear model of BMR against phenotype, age, age × phenotype and mass of intestine, liver, heart, kidneys and residue in TB$^-$ and control mice aged 52–84 days.
(Non-significant terms were successively removed; p to enter or remove was 0.10. β are partial correlation coefficients. Multiple $R^2 = 0.92$. Phenotype categories were not biased with respect to age ($F_{1,13} = 0.001$, $p = 0.98$). A model with age and (total) body mass, or body mass alone, as covariates, gave virtually identical results. Abbreviation: MS, mean square.)

variable	d.f.	MS	F	β	p
phenotype	1	2.22	15.1	—	0.004
age	1	0.74	5.04	−0.56	0.051
liver	1	0.86	5.82	0.55	0.039
heart	1	1.13	7.69	−0.50	0.022
residue	1	1.12	7.63	0.79	0.022
error	9	0.15			

immune defence. Lymphocyte-deficient mice compensate for their lack of adaptive defence by a higher activity of innate defence (Smelt *et al.* 2000; Bründler *et al.* 1996; Langhorne *et al.* 1995), but we found no other differences in growth or body composition that could explain the differences in BMR between phenotypes. Hence, our results indicate that an upregulated innate immune system carries a higher energetic cost than a combination of innate and adaptive defence.

It should be noted that we found a difference in BMR of *ca.* 10% between lymphocyte-deficient mice and controls, despite the fact that these mice were living in a laboratory environment. In a more natural environment, with a higher exposure to parasites and pathogens, the energetic benefits of the adaptive immune system should, if anything, be even higher.

What are the evolutionary implications of this finding? Our use of *μMT* and *TCR-β* mutant mice as a model of the ancestral state in vertebrate immune-defence evolution is based on the assumption that innate and adaptive immunity have been negatively genetically correlated and, consequently, that adaptive has, to some extent, replaced innate immunity during the evolution of vertebrates. This assumption is supported by quantitative genetic studies (Cheng *et al.* 1991). Our results therefore suggest that the energetic consequences of having an adaptive immune system should have favoured its evolution rather than constrained it. But what constrains the magnitude of adaptive defence? Autoimmunity is obviously a promising candidate (Behnke *et al.* 1992; Råberg *et al.* 1998), but its occurrence in natural populations undoubtedly demands further study before its role as a constraint can be assessed.

There is a possibility that the energetic cost of upregulation of innate defence in our lymphocyte-deficient mice is not representative of the cost of ancestral forms of the vertebrate immune system, because these knockout mice are not adapted to maintain this high level of innate defence. Alternative approaches to investigate the energetic consequences of variation in magnitude of innate and adaptive immunity include manipulation of the immune system by artificial selection and comparisons across species.

Because the benefit of defence in general is fairly obvious (i.e. resistance against infectious organisms), research into the ecology and evolution of immunological defence is currently focused on investigating the nature and magnitude of costs of defence (Read & Allen 2000). However, when it comes to the adaptive immune system, it is not as obvious why vertebrates have evolved this kind of defence in addition to the innate arm. One explanation is, of course, that a combination of innate and adaptive immune defence simply confers better protection against infectious organisms than innate defence alone, and that this is especially valuable in long-lived animals, such as vertebrates (Horton & Ratcliffe 1993). This explanation has been dismissed with the argument that there are also long-lived invertebrates and therefore no obvious reason why vertebrates should need an adaptive immune system (Burnet 1968). However, recent studies of knockout, mice have shown that deletion of particular components of the adaptive immune system often compromises resistance against specific pathogens (Kaufmann & Ladel 1994). Another explanation stems from the idea that the more self-antigens an organism has, the more diverse immune recognition mechanisms are required to distinguish self from non-self (De Boer & Perelson 1993). If so, the high complexity of vertebrates may have favoured the evolution of the extremely diverse receptors that characterize the adaptive immune system to avoid autoimmune responses (Borghans *et al.* 1999). Finally, the results of the present study imply that energy saving is another plausible factor favouring the evolution of adaptive immunity. The relative importance of these factors clearly deserves further study.

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