

Increased Levels of IgE and Autoreactive, Polyreactive IgG in Wild Rodents: Implications for the Hygiene Hypothesis

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Abstract

To probe the potential role of Th1 versus Th2 reactivity underlying the hygiene hypothesis, intrinsic levels of Th1-associated and Th2-associated antibodies in the serum of wild rodents were compared with that in various strains of laboratory rodents. Studies using rat lung antigens as a target indicated that wild rats have substantially greater levels of autoreactive, polyreactive immunoglobulin G (IgG), but not autoreactive, polyreactive IgM than do laboratory rats, both on a quantitative and qualitative basis. Increased levels of serum IgG and IgE were observed in both wild rats and wild mice relative to their laboratory-raised counterparts, with the effect being most pronounced for IgE levels. Further, wild rats had greater intrinsic levels of both Th1- and Th2-associated IgG subclasses than did lab rats. The habitat (wild versus laboratory raised) had a more substantial impact on immunoglobulin concentration than did age, strain or gender in the animals studied. The presence in wild rodents of increased intrinsic, presumably protective, non-pathogenic responses similar to both autoimmune (autoreactive IgG, Th1-associated) and allergic (IgE, Th2-associated) reactions as well as increased levels of Th1-associated and Th2-associated IgG subclasses points toward a generally increased stimulation of the immune system in these animals rather than a shift in the nature of the immunoreactivity. It is concluded that, at least to the extent that feedback inhibition is a controlling element of immunoreactivity, an overly hygienic environment may affect the threshold of both types of immune responses more so than the balance between the different responses.

Introduction

Allergy occurs more often in countries with the widespread use of modern medical practice [1, 2], having affected a staggering number of individuals: in the United States alone, it is estimated that 40–50 million people suffer from allergies [3]. The most widely accepted hypothesis explaining the development of allergy in developed countries is known as the 'hygiene hypothesis'. In addition, improved hygiene might also lead to the increased incidence of autoimmune disease in countries with the widespread use of modern medical practice [4]. The toll taken by this family of diseases is not trivial, affecting 8 million individuals in the United States alone. The idea that improved hygiene might account for

increased incidence of a Th1-type response (autoimmune disease) and a Th2-type immune response (allergy) is intriguing, suggesting that the mechanism underlying the hygiene hypothesis may not involve a preference for a Th1 versus a Th2 response, but rather may either (a) lead to loss of control of the balance between Th1 and Th2 responses or (b) may lead to hyper-responsiveness affecting both Th1 and Th2 responses. No evidence has been presented that would differentiate between these two possibilities.

The hygiene hypothesis [5] states that individuals living in industrialized societies with the widespread use of modern medicine have a poorly developed immune system because they are exposed to an unnaturally low amount of microbes, both pathogens and commensals. A

lack of exposure to various parasites may play a key role in this regard [6–9]. One potential mechanism underlying the hypothesis may be that a decreased antigen load leads to decreased stimulation of the immune system, in turn leading to decreased induction of suppressor or regulatory immune functions (e.g. Treg cells) that normally counterbalance immune activation in a potentially bystander fashion. Decreased suppressor immune function, in turn, could result in increased sensitivity to allergens and a potentially overactive phenotype. On the other hand, another potential mechanism underlying the hygiene hypothesis is that some organisms encountered under non-hygienic conditions might be recognized by the innate immune system's pattern recognition receptors in ways that lead directly to development of regulatory immune cells. Such downregulation of the immune system might be necessary to avoid immunopathology and could lead to hyporeactivity to a variety of allergens [10].

Regardless of the mechanism involved, the mechanisms underlying the aberrant immune response of hygienic animals and the details concerning the potential role of the immune system in the hygiene hypothesis are a matter of debate and have been discussed in detail by several investigators [5, 11, 12]. The lack of certainty regarding the details of the hygiene hypothesis and the difficulty in evaluating the hygiene hypothesis in human populations provide impetus for studies using animal models to probe mechanisms underlying the hypothesis.

Laboratory animals, unlike their wild counterparts, are shielded from many environmental antigens and from a wide range of pathogens, including ectoparasites, endoparasites and a variety of pathogenic viruses and bacteria. Thus, animals in the wild and in the laboratory can serve as a model for humans without modern medicine and humans with modern medicine respectively. Two species abundant in the laboratory setting and in the wild that are useful for this purpose are the house mouse (*Mus musculus*) and the common rat (*Rattus norvegicus*). In an initial study [13], we described the hypoproliferative nature of wild-rat-derived splenocytes compared to lab-rat-derived splenocytes in response to mitogen. The response of splenocytes to mitogen is not restricted to particular strains or even particular species, thus serving as an excellent marker for immune status that will not depend on genetic differences between wild rodents and laboratory rodents. Substantial differences in expression of T-cell markers on the lymphocytes from the two rodent populations were observed [13], although the implications of those differences are not clear.

In the initial study comparing the immune system of wild-raised and laboratory-raised animals [13], cytokine production by splenocytes in response to mitogen was evaluated in the hope of gaining information regarding the mechanism(s) underlying the hygiene hypothesis. Mitogen induced 100-fold greater IL-4 production and

10-fold less IL-2 and TNF- α production in wild-rat-derived splenocytes compared to lab-rat-derived splenocytes [13], potentially consistent with a greater propensity for Th2 responses relative to Th1 responses in wild rats compared to lab rats. On the other hand, IL-4 responses in the rat may not be uniquely associated with the Th2 response [14], and no significant effect of the environment on the production of IL-10, another Th2-associated cytokine, was observed [13]. Thus, the cytokine release profile of splenocytes from laboratory versus wild rats does not clearly point towards a difference in propensity for Th1 versus Th2 responses in these animals.

To further evaluate the effects of a hygienic laboratory environment versus a natural (wild), unhygienic environment on the immune system, herein we describe an initial test of the differences between the humoral immunological status of wild-caught versus laboratory-raised *M. musculus* and *R. norvegicus*. In particular, the difference in immune activation between wild and laboratory rodents as judged by the levels of autoantibodies, immunoglobulin (Ig) M, IgG and IgE in their serum was evaluated. The effects of gender, age and strain on antibody levels was evaluated to avoid, at least to some extent, potential confounding factors associated with differences in laboratory and wild animals that are not a direct result of their environment. The results indicate the presence of simultaneous and substantial effects of the environment on autoreactive, polyreactive IgG production and on IgE production. The results are consistent with the idea that animals without access to modern medicine have high levels of autoimmune-like and allergic-like processes that represent non-pathogenic, appropriate response to unknown factors in the environment. The findings indicate that an overly hygienic environment may affect Th1-related and Th2-related immune responses in a similar fashion. Thus, the hygiene hypothesis may involve a simultaneous change in a propensity for both Th1 and Th2 responses rather than dysfunction of factors controlling the balance between the two types of responses.

Materials and methods

Materials

Goat IgG specific for rat IgE (Fc specific) was purchased from Nordic Immunological Labs (Capistrano Beach, CA, USA). Affinity-purified, phosphatase-labelled goat antibodies specific for rat IgG (γ -chain specific); affinity-purified, unlabelled goat antibodies specific for rat IgG (γ -chain specific); affinity-purified, phosphatase-labelled goat antibodies specific for rat IgM (μ -chain specific); and affinity-purified, unlabelled goat antibodies specific for rat IgM (μ -chain specific) were purchased from Kirkegaard & Perry Labs (Gaithersburg, MD, USA). Human

serum albumin; Tween-20; affinity-purified, phosphatase-labelled goat antibodies specific for rat IgG (whole molecule specific); affinity-purified, unlabelled goat antibodies specific for mouse IgM (μ -chain specific); affinity-purified, alkaline phosphatase-labelled goat antibodies specific for mouse IgM (μ -chain specific); affinity-purified, unlabelled goat antibodies specific for mouse IgG (γ -chain specific); and affinity-purified, alkaline phosphatase-labelled goat antibodies specific for mouse IgG (γ -chain specific) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Affinity-purified, unlabelled goat antibodies specific for mouse IgE (ϵ -chain specific) and affinity-purified, alkaline phosphatase-labelled goat antibodies specific for mouse IgE (ϵ -chain specific) were purchased from Bethyl Laboratories (Montgomery, TX, USA). Four purified mouse anti-rat IgG subclass-specific antibodies (one each for rat IgG1, rat IgG2a, rat IgG2b and rat IgG2c) were purchased from BD Biosciences Pharmingen (Rockville, MD, USA). Peroxidase-labelled, monoclonal rat anti-mouse IgG and a ready-to-use ABTS Single Solution Chromogen/Substrate for horseradish peroxidase was obtained from Zymed Laboratories, Inc. (San Francisco, CA, USA).

Measurement of immunoglobulin levels

The relative concentrations of rat IgG, rat IgM, mouse IgG, mouse IgE and mouse IgM in serum was evaluated by ELISA as follows: 96-well Maxisorp plates (Nunc, Roskilde, Denmark) were incubated with 50 μ l/well of phosphate-buffered saline (PBS) containing 7.5 μ g/ml unlabelled antibody specific for the appropriate isotype of rat or mouse antibodies. Plates were incubated overnight at 4 ° C and then washed three times with 200 μ l/well of PBS. Plates were then blocked with 200 μ l/well of PBS containing 0.1% bovine serum albumin (BSA). Next, appropriately diluted rat or mouse serum (50 μ l/well) was added and incubated for 3 h. Each concentration was run in duplicate and sera were sufficiently diluted such that the readout from the ELISA was linearly proportional to the serum concentration. After incubation for 3 h at room temperature, wells were washed three times with 200 μ l/well of PBS and an alkaline phosphatase-conjugated antibody specific for the appropriate rat or mouse isotype was added to each well and incubated for 1 h at room temperature. Wells were then washed four times with PBS and the plates were developed using *p*-nitrophenyl phosphate as the substrate in a diethanolamine buffer. The absorbance at 405 nm was measured and the relative antibody concentration was taken to be the change in absorbance as a function of serum concentration. The concentration of rat IgE was determined in a similar manner, except that goat anti-rat IgE (Fc specific) was used as the primary antibody, and alkaline phosphatase-conjugated, goat anti-rat IgG (made against the whole molecule inclu-

ding the light chains, thus not isotype specific), was used as the conjugate.

The relative concentrations of rat IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) in serum were evaluated by direct ELISA as follows: 96-well Maxisorp plates (Nunc) were incubated with 50 μ l/well of PBS containing appropriately diluted rat serum (50 μ l/well). Each concentration was run in duplicate and sera were sufficiently diluted such that the readout from the ELISA was linearly proportional to the serum concentration. Plates containing diluted sera were incubated overnight at 4 ° C and then washed three times with 200 μ l/well of PBS. Plates were then blocked with 200 μ l/well of PBS containing 0.1% BSA. After incubation for 3 h at room temperature, wells were washed three times with 200 μ l/well of PBS and a peroxidase-labelled, monoclonal rat anti-mouse IgG antibody was added to each well and incubated for 1 h at room temperature. Wells were then washed and developed using a ready-to-use ABTS Chromogen Substrate solution for horseradish peroxidase. The absorbance at 405 nm was measured and the relative antibody concentration was taken to be the change in absorbance as a function of serum concentration.

The relative antibody concentration for samples from each experiment was normalized with respect to the relative concentration of antibody in a 'standard serum' determined in that experiment, so that results from various assays could be compared. Pooled, aliquoted and frozen sera from laboratory rats or laboratory mice were used as the standard sera.

Laboratory animals

Although ideal experiments might be considered in which inbred, laboratory strains are housed under 'wild-like' (natural) conditions and then compared with their genetically identical laboratory-housed counterparts, such experiments are not feasible given current practices involving laboratory animals. With this in mind, we utilized an experimental design aimed at comparing the immune system of animals in a natural environment with that of animals in a laboratory environment while controlling, to at least some extent, potentially confounding variables such as age, gender and, most importantly, genetic variability in wild-caught animals. For this purpose, multiple strains of laboratory animals were utilized, and tests were conducted on two species (mice and rats) rather than just one, when possible.

All experiments were approved by the Duke University Institutional Animal Care and Use Committee. Laboratory animals were housed in traditional barrier facilities operated by the Duke Laboratory Animal Resources (DLAR). Fifteen laboratory rats were used for the purpose of determining IgG, IgM and IgE levels. The strains used and the number of each strain is as

follows: male Fisher 344 (five), male WKY (two), male Sprague–Dawley (seven) and female Sprague–Dawley (one). The Fisher 344 and the WKY rats weighed approximately 250 g each and were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA) and/or from Charles River Laboratories, Inc. (Wilmington, MA, USA). The male Sprague–Dawley rats were retired breeders (approximately 500 g each) and the female Sprague–Dawley animal was a 240-g animal that had been pregnant.

The levels of rat IgG subclasses (IgG1, IgG2a, IgG2b and IgG2c) were evaluated in 30 laboratory rats. The strains used and the number of each strain is as follows: male Lewis (nine), male Fisher 344 (11), male Sprague–Dawley (one), male WKY (eight) and male Wistar–Firth (one). The Fisher 344 and the WKY rats weighed approximately 250 g each and were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA) and/or from Charles River Laboratories, Inc. (Wilmington, MA, USA). The male Lewis rats weighed approximately 450 g each, and the Sprague–Dawley and Wistar–Firth rats weighed about 300 g each.

For comparison of antibody levels in wild versus laboratory mice, seven strains of laboratory mouse [15, 16] were evaluated. This main portion of the study was not limited to one strain of laboratory mouse to ensure that observations were not due to unusual characteristics of one particular strain. The strains and the number of mice per strain are as follows: C3H (two), DBA (five), BDF-1(three), SJL (three), C57BL/6 (two), FVB (two), AKR (three) (20 total). All mice were female and were obtained from Charles River Laboratories. Blood was collected from these mice between the age of 2.5 and 6 months, and serum was separated from the blood and stored at -80°C until needed. Another series of experiments was conducted to determine whether the results might be affected by gender. For these experiments, blood was collected from BDF-1 mice ($n = 8$; four female, four male) mice at age 1, 2, 3, 4, 5, 6 and 8 months of age. Serum from these mice was also separated from the blood and stored at -80°C until needed. Because only small quantities of serum were collected, sera taken from mice of the same gender and age were pooled before freezing.

Serum from 1.7–2.7 month-old 'wild-derived inbred mice' was obtained from The Jackson Laboratories (Bar Harbor, ME, USA). Wild-derived inbred mice are descendants of captured wild mice and maintain behavioural characteristics reminiscent of wild mice rather than laboratory mice. However, the wild-derived mice have been raised in a 'clean' environment like other strains of laboratory mouse. The large number of genetic differences between wild-derived and common inbred laboratory mice make the wild-derived mice of particular interest for this study. Sera from four mice representing

four different wild-derived inbred strains were utilized. The strains and a brief description of each are as follows: strain CAST/EiJ (*Mus musculus castaneus*): the founders were trapped in a grain warehouse in Thonburi, Thailand and the strain has been maintained by The Jackson Laboratory since 1971; strain CZECH/Ei (*M. musculus*): the founders were trapped in Studenec, Moravia in 1978; strain MSM/Ms (*Mus musculus molossinus*): the founders were trapped in Mishima Prefecture, Japan in 1978; strain PERA/EiJ (*M. musculus*): the founders were trapped in Nana Village, Rimac Valley, Peru in 1961. All sera from wild-derived inbred mice were from females except the serum from the MSM/Ms mouse.

Wild animals

A first cohort of wild rats ($n = 28$) was caught in live traps and killed as part of an unrelated study evaluating parasitism in the animals. Wild rats were obtained from farms ($n = 18$) and from urban areas ($n = 10$) in North Carolina. Twenty of the wild rats were weighed on a scale to the nearest gram and the weights of the remaining eight rats were estimated by the investigators in the field. Following euthanization, blood was drawn and the serum was collected and frozen at -80°C until needed. Rats ranged in weight from less than 70 g to more than 400 g, and both males and females were used in the study. Serum from these rats was utilized in assays to evaluate IgM, IgG and IgE levels.

A second cohort of wild rats ($n = 30$) was, like the first cohort, caught in live traps and killed as part of an unrelated study evaluating parasitism in the animals. After elimination of the farm-based colony used in cohort 1 with poison by the farm owner, a feed-processing plant was selected as an additional source of wild rats. Wild rats were obtained from the animal feed-processing plant ($n = 14$) and from urban areas ($n = 16$) in North Carolina. The food source utilized by the wild rats in the feed plant was similar to that found on the farm, although there was a reduced risk of the wild animals being exposed to pesticides and anti-parasitic chemicals at the feed-processing plant. Rats were weighed on a scale to the nearest gram. Rats ranged in weight from 49 to 475 g, and both males and females were used in the study. Rats were killed and serum was collected as described above. Serum from this second cohort of wild rats was utilized in assays to evaluate IgG subclass levels.

Based on the established habits of wild rats and direct observations of the rat colonies from which wild rats were harvested for this study, rats captured in urban areas from both cohorts (cohorts 1 and 2) generally utilized faecal material from domestic dogs as a food source. On the other hand, wild rats from farms (cohort 1) and from animal feed-processing plants (cohort 2) utilized grain and other agricultural products as a primary source of

food. Although some of the wild rats may have been exposed to anti-parasitic drugs either mixed with the feed (farm-caught animals, cohort 1) or used to treat the domestic dogs from which their food was obtained (urban caught animals, cohorts 1 and 2), parasites were observed on wild rats regardless of their origin, and the location from which the rats were captured did not have a substantial impact on the results (see Results.)

Wild mice ($n = 10$) were caught in live traps in Kautzer, Wisconsin ($n = 6$) or in Muller, Wisconsin ($n = 4$) as part of an unrelated study evaluating the prevalence of monkey pox virus (all animals were negative). Mice weighed from 12.12 to 27.98 g (mean 18.00 g), with both males and females being used in the study. Mice were classified as juveniles, 'sub-adults' or adults by the investigator in the field based on the total length (tip of nose to fleshy tip of tail) of the animal. Sub-adults (7–8 in. long) had typical weights between 12 and 20 g, with adults (longer than 8 in.) typically weighing more than 18 g. Juveniles (less than 7 in. long, typically weighing below 14 g) were not utilized in the study and no analysis of their serum was performed. Half of the 10 animals used in the study were classified as adults and half as sub-adults. The animals were killed, blood was collected and serum was obtained and stored at -80°C until needed.

Binding of immunoglobulin to antigens as determined by immunoblotting

Rat lungs were explanted from WKY rats under anaesthesia. Blood was washed from the rat lungs by perfusion of the lungs via the pulmonary artery with Perfadex® (Vitrolife, Gothenburg, Sweden), a preservation solution containing dextran 40 as the colloid. Following removal of the blood, the rat lung was frozen in liquid nitrogen and pulverized, then solubilized with SDS sample buffer containing 10 mM dithiothreitol (20 μl sample buffer per mg tissue). The samples were boiled for 5 min, and 200 μl of sample was loaded on the large well of a

4–20% acrylamide gradient preparation gel (Ready Gel Tris-HCl; Bio-Rad, Hercules, CA, USA), separated electrophoretically and transferred to a PVDF membrane (Millipore Immobilon-P, Bedford, MA, USA). Molecular weight standards (Bio-Rad) were run in the standard well of the preparation gel. Membranes were cut into slices approximately 5 mm wide, blocked for 1 h at room temperature using 1.0 % BSA and 0.1 % Tween-20 in PBS (blocking buffer), and then incubated overnight at 4°C with rat serum diluted 1/400 in blocking buffer. Rat sera from laboratory rats ($n = 8$) and wild rats ($n = 8$) weighing greater than 125 g were selected at random for the study. Blots were washed three times with Tris-buffered saline and then incubated for 1 h with alkaline phosphatase-conjugated, affinity-purified goat antibody specific for rat γ -chain or rat μ -chain in blocking buffer. Finally, blots were washed three times with Tris-buffered saline and then developed with 0.33 mg/ml nitro blue tetrazolium and 0.165 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate in 100 mM Tris-HCl, 100 mM NaCl_2 and 5.0 mM MgCl_2 at pH 9.5.

Results

Immunoglobulin levels in wild and laboratory rats

The levels of IgM, IgG and IgE in the serum of wild rats were evaluated by ELISA and compared with a similar data from laboratory rats. For the analysis, wild rats weighing at least 50% as much as the smaller laboratory rats (~ 250 g) were used for the comparison. As shown in Fig. 1 and in Table 1, wild rats weighing more than 125 g had substantially more IgM, IgG and IgE in their serum than did laboratory rats (weight >250 g). Even smaller wild rats (<125 g) had greater average immunoglobulin levels in their serum than did the laboratory rats (Fig. 1), which were all larger. Using sera obtained from a separate cohort of wild rats, the level of IgG1, IgG2a, IgG2b and IgG2c was evaluated (Fig. 2). Large wild rats (>125 g) had significantly ($P < 0.05$) more of

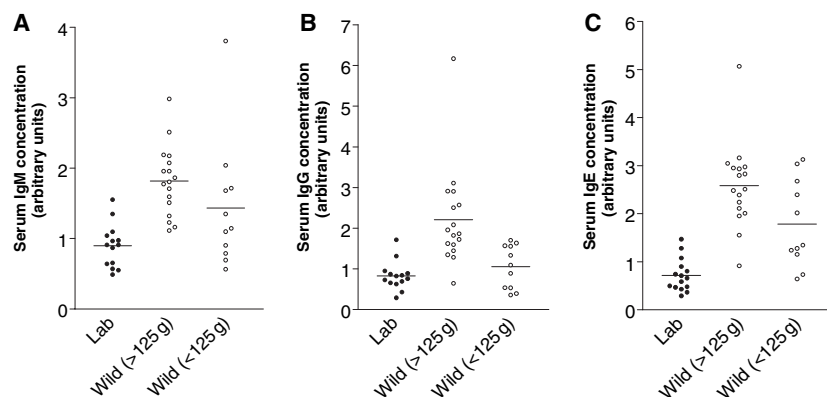


Figure 1 Immunoglobulin levels in laboratory and wild rats. Immunoglobulin levels were determined by ELISA and are expressed in arbitrary units as described in Materials and methods. Laboratory rats weighed 250 g or more with the exception of one 240 g animal, and the wild rats were arbitrarily divided into two groups (greater than and less than 125 g) for the sake of comparison. The mean value of each group is shown by the line.

Table 1 Effect of habitat (wild versus laboratory raised) on immunoglobulin concentrations (arbitrary units) in rats and mice

Species	Isotype	Mean $\pm$ SE (<i>n</i>)		Ratio (wild/lab levels)	P-value	
		Laboratory	Wild		<i>t</i> -test	<i>F</i> -test
Rat	IgM	0.90 $\pm$ 0.08 (14)	1.82 $\pm$ 0.12 (17)	2.03	<0.0001	0.0458
Rat	IgG	0.83 $\pm$ 0.09 (14)	2.21 $\pm$ 0.29 (17)	2.27	0.0003	<0.0001
Rat	IgE	0.72 $\pm$ 0.09 (15)	2.59 $\pm$ 0.21 (17)	3.59	<0.0001	0.0005
Mouse	IgM	0.74 $\pm$ 0.07 (24)	0.96 $\pm$ 0.19 (10)	1.30	0.2024	0.0200
Mouse	IgG	6.22 $\pm$ 1.30 (24)	15.36 $\pm$ 4.32 (10)	2.47	0.0114	0.0015
Mouse	IgE	2.08 $\pm$ 0.50 (24)	25.74 $\pm$ 5.90 (10)	12.4	<0.0001	<0.0001

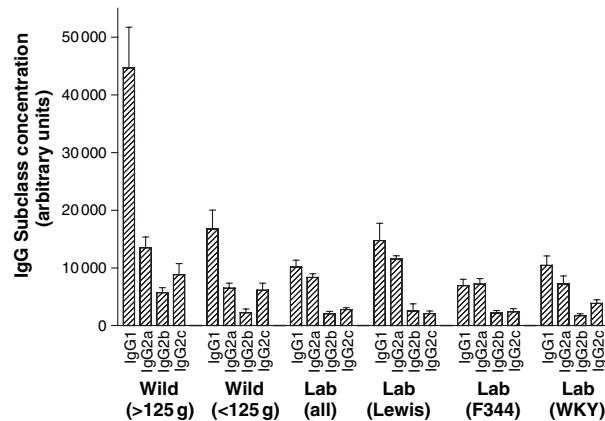


Figure 2 IgG subclass levels in wild and laboratory rats. IgG subclass levels were determined by ELISA and are expressed in arbitrary units as described in Materials and methods. The arbitrary units used to describe IgG subclass levels are unrelated to those used to describe IgG or other isotype levels. Data from large wild rats ($n = 19$), small wild rats ($n = 11$), all laboratory rats, regardless of strain ($n = 30$), Lewis rats ($n = 9$), F344 rats ($n = 11$) and WKY rats ($n = 8$) are shown. The mean values (bars) and standard errors are shown.

all subclasses of IgG than did the lab rats used for comparison (results of all strains averaged together). This result suggests that wild rats have higher levels of intrinsic Th1-type immune activity (as indicated by increased levels of IgG1) and Th2-type immune activity (as indicated by increased levels of IgG2b) than do laboratory rats.

IgG subclass usage varied somewhat between Lewis, F344 and WKY rats. However, some aspects of the IgG subclass usage in the wild rats were markedly different than that of laboratory rats, regardless of the strain of laboratory rats. Of note is the observation that the relative utilization of IgG1 in large wild rats (62% of total IgG in those rats) was greater than that observed in lab rats (44% of total IgG), whereas utilization of IgG1 (53% of total IgG) in smaller lab rats (<125 g) fell between that of large wild rats and lab rats. The relative increase in utilization of IgG1 in the wild rats was offset by a decrease in utilization of IgG2a (19% of total IgG in large wild rats, 21% in small wild rats and 36% in

laboratory rats). Given the importance of various cytokines in class switching, these data might suggest that wild rats and lab rats possess different cytokine milieus associated with their respective humoral responses. On the other hand, differences in genetics or exposure to antigen may also account for the observed differences in subclass usage.

There was a significant correlation between the weight and the concentration of IgG ($r^2 = 0.32$, $P = 0.0053$) in the serum of wild rats weighing up to 225 g. There was also a significant correlation between the weight and the concentration of IgE ($r^2 = 0.21$, $P = 0.029$) in the serum of rats weighing up to 225 g. There was no significant correlation between weight and immunoglobulin concentration in the serum of rats weighing over 225 g, and there was no significant correlation between IgM concentration and weight in any of the rats. Although significant correlations were observed, the correlation coefficients were relatively small (0.32 and 0.21 for IgG and IgE respectively), indicating, perhaps not surprisingly that factors other than size have a profound effect on immunoglobulin concentration in wild rats.

No significant difference in serum concentrations of IgM, IgG or IgE was observed between wild female and wild male rats ($P = 0.44$, 0.56 and 0.75 respectively). Thus, environment or perhaps genetic factors apparently had a more substantial effect on immunoglobulin level than did gender. No effect of the environment was observed when different wild environments were compared, as there was no significant difference between levels of IgM, IgG or IgE in the serum of larger wild rats (>125 g) captured in a rural environment compared with those captured in an urban environment ($P = 0.66$, 0.056 and 0.40 respectively). Insufficient numbers of smaller (<125 g) rats were captured in an urban environment to make a comparison between smaller urban and smaller rural wild rats.

Immunoglobulin levels in wild and laboratory mice

The levels of IgM, IgG and IgE in the serum of wild mice were evaluated by ELISA and compared to levels found in laboratory mice. As shown in Fig. 3 and in

Figure 3 Immunoglobulin levels in laboratory and wild mice. Immunoglobulin levels were determined by ELISA and are expressed in arbitrary units as described in Materials and methods. Immunoglobulin levels in the 24 laboratory mice were evaluated when the mice were between 1.7 and 6 months of age. The mean value of each group is shown by the line.

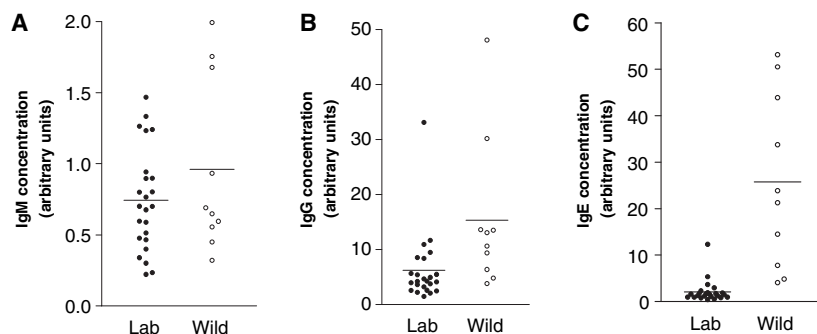


Table 1, wild mice had substantially more IgG and IgE in their serum than did laboratory mice aged 1.7–6 months. This effect was much more pronounced with IgE, wild mice having 12.4-fold more IgE than their laboratory counterparts. In contrast, there was no significant difference in IgM levels between the wild and laboratory mice. The greater levels of IgG and IgE in the wild mice were despite the fact that half of the wild mice were classified as ‘sub-adults’, with four wild mice weighing <16 g. All laboratory mice, in contrast, were adults of known age (7 weeks of age or greater; corresponding to a weight of >17 g). The older age of the laboratory animals relative to the wild animals cannot account for the relatively lower level of immunoglobulin in the laboratory rodents, given the effect of age on immunoglobulin concentration in mice <1 year of age. Thus, differences between the ages of the wild and the laboratory mice do not account for the observation that wild mice have higher immunoglobulin concentrations in their serum than do laboratory mice.

Of particular interest were the immunoglobulin levels in wild-derived, inbred laboratory mice. These mice are genetically and behaviourally more related to wild mice than to the common strains of laboratory mice. Four wild-derived mice with four distinct backgrounds were evaluated as described in Materials and methods. The immunoglobulin levels in these four mice were not high, with the IgE levels in all four mice being below the mean levels for all the laboratory mice (mean for wild-derived laboratory mice was 2.08 arbitrary units, see Fig. 3C). This finding indicates that it is the environment rather than genetic factors that are the major contributors to the differences in immunoglobulin levels observed in this study. In further support of this idea, levels of IgE in all laboratory mice, regardless of strain, were less than the mean IgE concentration in wild mouse serum (Fig. 3C). Thus, although insufficient numbers of mice from each laboratory strain were evaluated to facilitate comparison between laboratory strains, it was evident that immunoglobulin levels in the serum of laboratory mouse strains were substantially less than that in wild mice.

Effect of age and gender on immunoglobulin levels in laboratory mice

Factors such as age and gender may affect immunoglobulin levels in rodents, thus possibly confounding the present study if not taken into consideration. Immunoglobulin levels did increase as a function of age in some but not all strains of mice used in this study. For example, the concentration of serum IgM increased in C3H mice as they aged from 2 to 11 months, whereas the concentration of serum IgM in SJL mice did not increase between 2 and 11 months of age. However, the greatest level of IgE found in any of the laboratory mice at any age was still lower than the mean level of IgE in wild mice. Thus, although there may be some effect of age on immunoglobulin concentration in adult mice that effect is eclipsed by the effect of other factors such as environment in the population studied.

The effect of gender on immunoglobulin levels was evaluated in one laboratory strain of mouse (BDF-1 strain, four males and four females tested monthly for 8 months starting at 1 month of age). No consistent effect of gender on immunoglobulin concentration was observed, at least in the particular strain of laboratory mouse in which it was evaluated (data not shown). Further, the lack of a substantial effect of gender on immunoglobulin levels was also observed in wild rats, suggesting that any potential effect of gender on immunoglobulin concentrations in rodents might be small and may require evaluation of a substantial sample size for quantification. With this in mind, it seems likely that factors such as gender and age do not account for differences in immunoglobulin concentration observed between laboratory and wild rodents.

Absolute levels of IgE in hygienic and unhygienic populations

Determination of absolute levels of immunoglobulin in rodents is frequently dependent on the assay used [17], and thus the data we have presented are expressed as relative values. However, a comparison of absolute values

between species may be of interest in the case of IgE, as this isotype is ubiquitously associated with the allergic response. Using commercially available monoclonal IgE as a standard, the relative concentrations of IgE in the serum of rats and mice were converted to standard concentrations and compared with levels in humans (Fig. 4). Of note is the observation that absolute IgE levels in rats are considerably higher than levels in humans and mice, regardless of the environment. The effect of an overly hygienic environment had a similar effect on levels of human and mouse IgE, resulting in a greater than 90% reduction in serum IgE concentration in both species (Fig. 4, inset). In contrast, the overly hygienic environment resulted in a less than a 70% drop in IgE levels in the rat. It remains unknown whether differences in the environments account for this difference between rats and the other two species, or whether there are fundamental

differences between the various species that account for this difference.

Autoantibody levels in wild and laboratory rats

A population of B cells, termed B-1 B cells, exists in all normal individuals that produce autoreactive, polyreactive IgM, which may be involved in innate immunity [18–20]. The non-pathogenic autoantibodies produced by these cells frequently bind to autologous antigens that are not exposed on normal, healthy tissue but which may be exposed upon cell lysis. Of note is the observation that these non-pathogenic, autoreactive, polyreactive antibodies are typically IgM, not IgG [18, 21], although studies to date have focused on hygienic populations.

One of the hallmarks of autoimmune disease is the development of IgG antibodies that react with autologous tissue. These antibodies are likely produced, at least in some cases, as a result of pathological changes in the B cells that normally produce non-pathologic, polyreactive, autoreactive IgM. Following aberrant class switching and affinity maturation, these B cells can produce high-affinity IgG that reacts with healthy tissue, thus leading to autoimmune pathology [18]. To determine to what extent class switching and affinity maturation of such B cells might happen in wild rats, we evaluated the serum of wild rats for the presence of autoreactive antibodies and compared the results with those obtained using serum from laboratory rats.

Binding of rat IgM to rat lung antigens as demonstrated by Western blots was similar regardless of whether wild-rat-derived serum or laboratory-rat-derived serum was used as a source of IgM (Fig. 5, top panels). In stark contrast, rat IgG from wild rats bound substantially more antigens than did rat IgG from laboratory rats (Fig. 5, bottom panels, Fig. 6A). It is likely that the antibodies recognize 'cryptic epitopes' on the rat lung antigens, which are not exposed in the intact rat lung but which are exposed on the Western blot. The increase in IgG reactivity from antibodies presumably derived from B-1 B cells was not limited to autoantibodies, as IgG from wild rats reacted with dinitrophenol (a common antigen recognized by non-pathogenic autoreactive/polyreactive antibodies) to a significantly greater degree than did IgG from lab rats (Fig. 6B). Further, immunoblots using antigens derived from laboratory rat food yielded results very similar to those obtained using rat lung antigens: wild-rat-derived IgM and laboratory-rat-derived IgM has equivalent reactivity with rat food antigens, whereas wild-rat-derived IgG had substantially greater reactivity with those antigens than did laboratory-rat-derived IgG (data not shown). These data point towards a heightened IgG response from the B-1 B cell population, which produces autoreactive, polyreactive antibodies and which can be associated with autoimmune disease in hygienic humans.

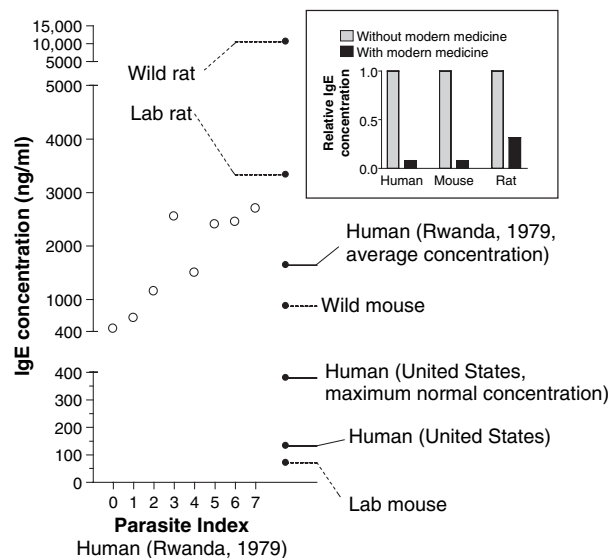


Figure 4 Mean IgE levels in population of humans, rats and mice both without (wild animals and Rwandan tribes people in 1979) and with (laboratory animals and Duke University hospital personnel and patients) modern medical care. The mean IgE levels of the groups of rats and mice (dashed lines) as determined in this study are shown. Levels of IgE in humans have been previously reported [43, 44]: the Rwandan population consisted of Hutus (farmers of Bantu origin) and Twas (a Pygmoid group in large part peasants, potters or servants of farmers at the time of the study). In that study, patients were divided into groups based on the parasite load (parasite index) based on a semiquantitative analysis of parasites in faecal samples [43]. The levels of IgE in Rwandan individuals as a function of their parasite index is shown (open circles). Individuals in the United States selected for the study were hospital personnel and patients attending the Duke Pediatric Outpatient and Cardiac Clinics. Individuals with recurrent infection, parasitism, atopic disorders or autoimmune diseases were excluded from that population [44]. The maximum 'normal' level of serum IgE in the United States is less than 400 ng/ml IgE [45]. The relative levels of IgE normalized with respect to the levels found in population without access to modern medicine are shown in the inset.

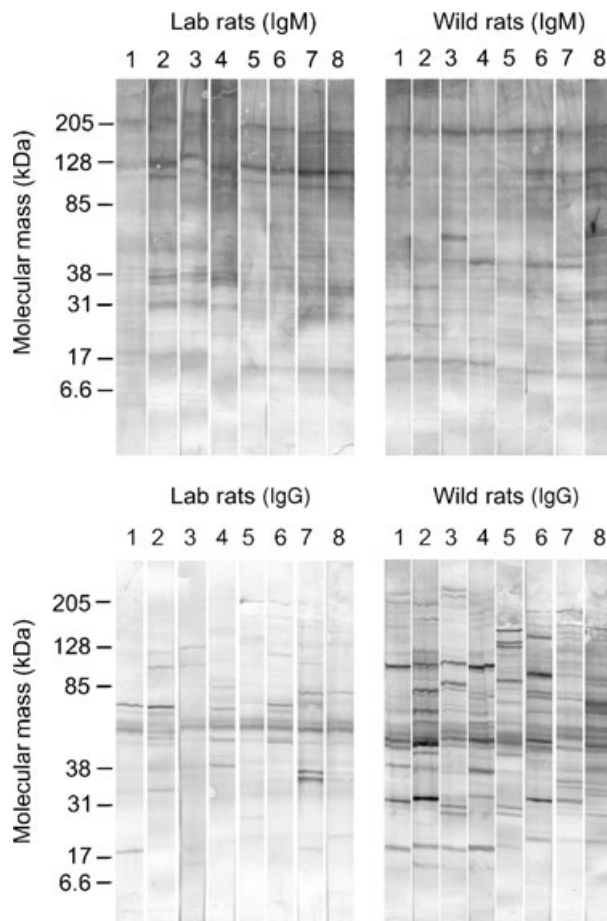


Figure 5 Reactivity of serum IgG and IgM from wild and laboratory rats with rat lung antigens. Rat lung antigens were separated by electrophoresis and transferred to membranes, and binding of IgM or IgG to those antigens was detected by immunostaining as described in Materials and methods. Eight rats from each group were selected at random for the study, with the only stipulation being that all wild rats used in this part of the study weighed more than 125 g. The development time for the blots was between 30 and 35 min for both the IgM- and the IgG-stained blots.

Discussion

The results presented suggest simultaneous and substantial effects of the environment on autoreactive IgG production and IgE production. While these reactions lead to autoimmune disease and allergy, respectively, in hygienic populations, the reactions apparently represent a non-pathogenic, protective response to the environment by the wild, unhygienic animals. Indeed, it is expected that the targets of the autoreactive IgG and the IgE in hygienic populations will be substantially different than the targets of the same antibodies in unhygienic animals. For example, autoreactive, polyreactive IgG in unhygienic individuals may bind to 'cryptic epitopes' that are not exposed to healthy tissue and to environmental antigens, in a manner similar to that of autoreactive, polyreactive

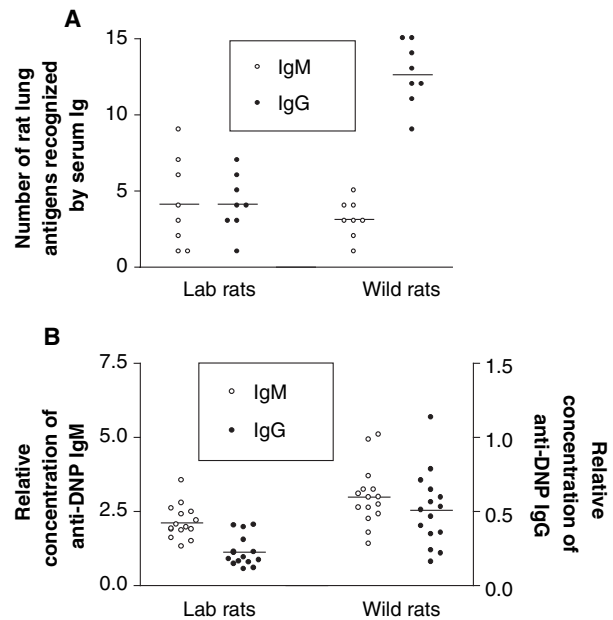


Figure 6 (A) Number of denatured rat lung antigens recognized by serum IgG and IgM from wild and laboratory rats ($n = 8$ for both groups). Bands in Fig. 5 for which the band intensity exceeded 15% of the maximum intensity (>29 of 195 optical density units) were counted. Each point on the graph represents the number of bands on a single lane in Fig. 5, corresponding to a single animal. (B) The concentration of anti-dinitrophenol (DNP) IgM and IgG in the serum of wild and laboratory rats as determined by ELISA. A significant ($P = 0.007$) but small (40%) increase in anti-DNP IgM and a 2.2-fold increase ($P = 0.0004$) in anti-DNP IgG were observed. Only the serum of those wild rats weighing more than 125 g was evaluated in this part of the study.

IgM in normal, hygienic individuals [19]. Autoreactive IgG in hygienic populations, on the other hand, can bind avidly to intact cells. In a parallel fashion, IgE in unhygienic animals bind to parasite antigens and are thereby protective, whereas the same antibodies in hygienic animals bind to abundant but harmless environmental antigens and lead to allergic disease.

If, as might be expected intuitively and as experimental evidence would suggest [22–26], inhibitory feedback mechanisms are associated with immune function, the present findings predict that the overly hygienic animals in the laboratory will simultaneously exhibit an increased propensity for Th1-related (e.g. autoimmune) and for Th2-related (e.g. allergic) immune responses compared with wild animals. If correct, this would argue that it is not a dysregulation of the balance between Th1 versus Th2 responses that underlies the hygiene hypothesis, but rather parallel affects of the environment on both types of responses, creating a hyper-responsive immune surveillance independent of the type of response.

The study design was aimed at assessing the role of gender and age, and, to a degree, the effect of genetics on immunoglobulin levels. While gender and strain did

not appear to be a major determinant of immunoglobulin levels in either rats or mice, the age of the animals was a significant factor. Smaller and thus younger wild rats (<125 g) and very young laboratory mice (1 month old) had substantially lower immunoglobulin levels than did older wild rats and laboratory mice respectively. However, even young wild rats and wild mice exhibited higher levels of immunoglobulin than did their older laboratory counterparts. Thus, although age, especially shortly after birth, is a critical factor determining immunoglobulin levels, this factor rapidly diminished in importance during the early development of rodents.

Despite the indications that environment rather than genetics is the primary factor determining immunoglobulin levels in the rodents studied, there was some overlap in immunoglobulin levels in some cases between wild and laboratory animals, and the role of genetics in determining immunoglobulin levels cannot be assessed accurately without further experiments. This statement is particularly applicable to the rat, as laboratory-raised, wild-derived strains of rat are not currently available.

The finding that wild rodents have substantially more IgE in their serum than do their laboratory counterparts is consistent with the observations that parasites elicit an IgE response [6–9] and that wild rodents have numerous parasites [27, 28]. Whether the same environmental factors that cause increased levels of IgE production also cause increased levels of IgG production, including auto-reactive IgG production, remains unknown. The answer to this question will await further experiments and will likely provide great insight into research aimed at addressing health problems associated with the hygiene hypothesis.

The effects of laboratory and natural (wild) environments on the immune system of rodents may serve as a model for not only the effect of hygiene on the human immune system, but for the potential effect of other factors on the immune system as well. Other than hygiene, there are numerous differences between societies with and those without widespread use of modern medical practices, any of which could account for the increased incidence of allergy (or autoimmune disease) in industrialized countries [29]. For example, greatly reduced physical activity or a lack of exercise is associated with industrialized countries and has a profound effect on the immune system [30–33]. As another example, it has been hypothesized that the processing and importation of food in industrialized societies may have a detrimental impact on the development of the immune system's relationship with its environment, leading to the increased incidence of allergy [34]. Consistent with this idea is the fact that oral administration of antigen tends to be toleragenic [35–37]. The factors associated with industrialized societies such as improved hygiene, inadequate stress release venues, inadequate exercise and ingestion of processed,

hygienic and imported food are also present to at least some extent under laboratory housing conditions for rodents. Thus, we anticipate that experiments utilizing rodents living in laboratory versus natural habitats might potentially be used to evaluate the immunological consequences of a number of conditions that characterize hygienic societies.

Another factor that differs between industrialized societies with the widespread use of modern medicine and traditional societies without such medicine is the level of mental stress: levels of chronic mental stress are thought to be greater in industrialized countries for a variety of reasons [38, 39] and the effects of mental stress on the immune system are well documented [40, 41]. The effect of stress may be particularly complex in this rodent model, as has been discussed previously [13]. The wild rats used in this study undoubtedly experienced substantial acute stress as a result of the trapping process, which may have affected the immune status of the animals. However, it seems unlikely that trapping-induced stress had a profound effect on the present results. First, acute stress, unlike chronic stress, generally causes an increase in immune sensitivity [42], in contrast to the relatively hypoproliferative cellular response observed in the wild rats [13]. Secondly, it is not expected that immunoglobulin levels would be substantially altered within the time frame of the procedure; some wild rats were sacrificed within 30 min of capture and all wild rats were sacrificed within 12 h of capture. Nevertheless, changes in stress levels, such as a shift from acute stress to chronic stress, may play a profound role in the incidence of allergy and autoimmune disease, and may play a role in this model. It seems likely that the wild animals experience more frequent acute stress than do the laboratory animals, although the relative levels of chronic stress between the two groups of animals are unknown.

The results presented herein suggest that future experiments comparing wild-raised and laboratory-raised rodents are worthwhile. Of critical and immediate importance is the evaluation of the susceptibility of wild-raised animals to allergy and autoimmune disease. One interesting but long-term line of inquiry will be evaluation of the factors that affect the propensity for wild-raised versus laboratory-raised animals to exhibit allergic or autoimmune reactions.

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