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The effects of obesity and fatty acids on the feline immune system

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Abstract

Obesity is a rising problem in cats. It is a risk factor for several diseases and has been linked to impaired immunity. The goal of this study was to determine the effect of body composition and effects of diet on immune function in cats. Twenty-eight short-term obese and 12 lean cats with equal gender distribution were evenly and randomly divided into two groups which were either fed a diet containing saturated (SFA) or long-chain $n-3$ polyunsaturated fatty acids (3-PUFA) for a period of 6 months prior to testing. Blood was collected by venipuncture from the jugular vein. Blood samples were analyzed in a double blind fashion. A complete blood count was performed and lymphocyte distribution was examined by flow cytometric analysis with specific fluorescein-conjugated subset markers. Immune function was measured as follows: the proliferative activity of different cellular fractions was tested with polyclonal mitogens such as lipopolysaccharide (LPS), phytohaemagglutinin (PHA), phorbol 12-myristate 13-acetate (PMA), Ca ionophore, and concanavalin A. Innate immune functions assessed were phagocytosis and natural killer cell (NK) cytotoxicity.

A similar immune innate and adaptive immune response was elicited regardless of diet or body condition. However, there was no correlation between body condition, diet, and any of the quantitative and qualitative functional responses of the immune system. We conclude that short-term obesity and the fatty acid composition of the diet do not alter immune responses in cats.

Keywords: Immunology; Obesity; Nutrition

1. Introduction

Obesity is increasing at a rapid rate in pets and people. The incidence of obesity today in the cat is approximately 35% (Lund et al., 2005). Obesity has been associated with a wide variety of diseases in people and pets including diabetes mellitus, cardiovascular disease, hypertension, dyslipidemia, gallstones and certain forms of cancer (Samartin and Chandra, 2001; Stallone, 1994; Wolf and Colditz, 1996). In addition to these conditions, clinical and epidemiological studies have shown that both, the incidence and severity of a number of infectious diseases are more prevalent in obese human beings when compared with lean counterparts. Wound healing has been shown to be impaired after surgical procedures (Fasol et al., 1992; Moriguchi et al., 1998; Plotkin et al., 1996) in obese people and they exhibit a lower antibody response than age-matched lean subjects when vaccinated against hepatitis B vaccine (Gottschlich et al., 1993; La Cava and Matarese, 2004). Obesity has also been linked to impaired immunity in rodents (Lund et al., 2005; Moretta et al., 2002; Moriguchi et al., 1998). It has

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also recently been suggested that obese dogs and cats (German, 2006) have decreased immune function but specific studies assessing their immune function have not been performed. In one study, obesity in cats was shown to be a predisposing factor for the development of blowfly myasis (ulcers) caused by *Calliphora erythocephala* larvae (Rodriguez and Perez, 1996). In spite of the suggestive evidence reporting a link between impaired immunity and obesity, direct comparisons of specific immune responses have not been forthcoming and therefore, it is not clear if cats immune function is also impaired as a result of body composition.

Additionally, diet is known to modulate immune function and specific food components are said to either enhance or diminish resistance to parasitic or bacterial infections. Polyunsaturated fatty acids, specifically long-chain $n-3$ PUFA, have been reported to dampen inflammatory responses (x) (Calder, 2004; Plat and Mensink, 2005). The present study was performed to compare immune responses in obese and lean cats in an effort to determine if cats have a similar immune response based on body composition as reported in other species. Furthermore, the effect of diet (SFA vs. 3-PUFA) on immune function was also investigated.

2. Materials and methods

2.1. Animals and diets

Forty adult neutered purpose-bred cats (Harlan Sprague Dawley, Madison, WI) were used. There were 28 obese and 12 lean cats of equal gender distribution. Obesity was induced by allowing ad libitum food intake and the obese cats have been described previously in detail (Wilkins et al., 2004). The cats were maintained at the University of Georgia College of Veterinary Medicine Animal Care Facility, using standard colony conditions. Cats were housed separately and were provided unlimited access to water. Animal studies were approved by the University of Georgia Animal Care and Use Committee and conducted in accordance with guidelines established by the Animal Welfare Act and the National Institutes of Health. It was determined that the cats were healthy on the basis of results of physical examination and clinical laboratory tests. All cats were used to being handled daily. Cats were evenly and randomly allocated to one of two diets (Table 1), 3-PUFA and SFA. The diets were fed for 6 months before the blood samples were taken. The weight (kg) of the lean cats was 3.8 ± 0.4 in 3-PUFA and 3.7 ± 0.6 in SFA; the weight of the obese cats was 5.5 ± 1.2 in 3-PUFA and 5.6 ± 0.8 in SFA ($p < 0.003$, lean vs. obese). Blood was sampled by venipuncture from the jugular vein and processed immediately. The cats were allocated to four groups of 10 cats each in random fashion. Always only one group of cats was tested per day.

Table 1
Percent of moisture, protein, ash, calculated carbohydrate, calculated calories per gram, fat, and percent of fatty acids in the diet containing $n-3$ polyunsaturated fatty acids (3-PUFA) or saturated fatty acids (SFA)

	3-PUFA	SFA
Moisture	8.41	9.28
Protein	34.40	34.30
Ash	6.45	6.38
Carbohydrate	31.90	32.30
Cal/g	4.33	4.28
Fat	18.40	18.10
14:0	3.53	2.01
14:1	0.20	0.39
15:0	0.38	0.33
16:0	21.00	22.50
16:1 $n-7$	6.33	4.20
17:0	0.55	0.86
18:0	7.33	11.90
18:1 $n-9$	25.70	34.90
18:1 $n-7$	2.33	1.75
18:2 $n-6$	11.40	11.10
18:3 $n-3$	1.01	0.68
18:3 $n-6$	0.17	0.11
20:0	0.22	0.18
20:1	0.77	0.32
20:2	0.12	<0.01
20:3 $n-6$	0.20	0.13
20:4 $n-6$	0.66	0.46
20:5 $n-3$	1.01	0.68
22:2	0.62	<0.01
22:5 $n-3$	0.83	0.12
22:6 $n-3$	4.72	0.46
24:0	<0.01	<0.01
24:1	0.17	<0.01
Unknown	5.02	2.48

2.2. Cell preparation

A complete blood count (CBC) was performed by the clinical pathology laboratory at the College of Veterinary Medicine, University of Georgia. The counts were conducted in an automated hematology analyzer and verified by differential staining and microscopy.

2.3. Fractionation of peripheral blood leukocytes (PBL)

Sterile samples of 5 ml whole blood were diluted with RPMI 1640 (Sigma, St. Louis, MO) containing 10% fetal bovine serum to 10 ml and layered over

Histopaque (Sigma, St. Louis, MO) before centrifugation at $400 \times g$ for 30 min. The PBL fraction was aseptically collected, washed and counted for use in experiments to study distribution and proliferation of lymphocytes as well as natural cytotoxicity.

2.4. Preparation of peripheral blood phagocytes

Phagocytic components of blood were prepared by osmotic shock lysis of red blood cells. Briefly, the pellet obtained by Histopaque centrifugation was diluted in cold water for 30 s before addition of 1/10 volumes of 10× phosphate buffered saline (PBS) with 5% glucose. The cells obtained after washing were resuspended in glucose containing buffer and tested in phagocytosis and oxidative burst assays in accordance with the manufacturer's directions.

2.5. Distribution of lymphocytes subsets in peripheral blood by flow cytometry

PBL were analyzed by flow cytometry using an EPICS XL-MCL analyzer (Beckman, Fullerton, CA). Cells were incubated in PBS containing 1% (w/v) bovine serum albumin (BSA) and 0.05% sodium azide (PAB) before staining with primary antibody followed by addition of fluorescein isocyanate (FITC) conjugated anti-rabbit IgG (Sigma, St. Louis, MO). Control rabbit IgG and appropriate conjugate controls were used to determine the negative cell populations. A panel of commercially available cell surface markers was used to assess frequency of the different populations. The following reagents were used: B cell marker: anti-CD21 (Serotec, Raleigh, NC); Pan-T cell marker: anti-CD5 (VMRD, Inc. Pullman, WA); CD4 T cell marker (Southern Biotechnology Associates Inc., Birmingham, AL); CD8 T cell marker (Southern Biotechnology Associates Inc., Birmingham, AL); NK cell marker: mAb 5C6 (Affinity Bioreagents, Inc. Golden, CO). Data are shown as the percent of positive cells in the PBL population.

2.6. Analysis of lymphocyte proliferative responses

PBL were plated in 96-well sterile plates at different concentrations in the presence of media, or media containing three different concentrations of LPS, Concanavalin A, PMA/Ca ionophore, PHA or pokeweed mitogen (PWM). Replicate plates were frozen at times 0 h, 24 h and 48 h. Proliferation was measured by the Cy QUANT cell proliferation kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions in a fluorescent microplate reader.

2.7. Natural cytotoxicity

PBL were tested for NK cell activity by the standard Cr 51 release assay for 4 h using cat feline cells lines as targets (FL74), kindly provided by Dr. Wayne Tompkins at the North Carolina State University. Three different effector to target cell ratios (E:T) were tested at 100, 50 and 25:1. Percent specific release (SR) was calculated as follows: cpm in test well − cpm in spontaneous release well/cpm in total release well − cpm in spontaneous release well × 100.

2.8. Phagocytosis assays

Phagocytic cells were incubated with fluorescein-labeled *E. coli*, or *S. aureus* for 30 min in a 37 °C water bath in the presence of opsonizing cat sera. The cells were analyzed by flow cytometry for percent uptake of the bacteria before and after trypan blue quenching of extracellular fluorescence.

2.9. Statistical analysis

Results of the experiments were calculated and analyzed without prior knowledge of the identity of the grouping. All data were analyzed by use of computer software (Prism Software, GraphPad Software Inc., San Diego, CA). The data are expressed as means ± S.D. unless otherwise stated. The significance of differences of means between groups was evaluated by ANOVA. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Differential white blood cell analysis

The analysis of white blood cell counts and differential blood cell counts revealed no significant differences in cats from each group (Table 2). Diet had no significant effect in the total absolute number of white cells, nor in the number of peripheral blood lymphocytes or in the neutrophil numbers.

3.2. Lymphocyte subset analysis by flow cytometry

Although the absolute number of white cells was found to be similar in all groups of cats, it was of interest to determine if subsets of lymphocyte populations had different frequencies depending on the diets the animals were fed. Several commercial feline lymphocyte subset markers were used in flow cytometric analysis of white cell populations to obtain the relative percentages of

Table 2
White blood cell count (WBC), segmented neutrophil (SEGS) and lymphocyte (LYMPHS) count in two groups of 6 lean and 12 obese cats fed either a diet containing $n-3$ polyunsaturated fatty acids (3-PUFA) or saturated fatty acids (SFA)[a]

Groups	Mean WBC[a]	Mean SEGS[a]	Mean lymphs[a]
Lean 3-PUFA	9.95 + 5.08	7.55 + 4.62	1.82 + 0.75
Lean SFA	8.00 + 2.56	5.37 + 2.39	1.92 + 0.35
Obese 3-PUFA	8.86 + 2.27	6.03 + 2.01	2.20 + 1.34
Obese SFA	10.00 + 2.63	6.17 + 1.48	3.00 + 1.65

[a] Numbers are $\times 10^3$/100 μl of whole blood. No significant differences in the numbers of white cells (WBC), neutrophils (SEG), or lymphocytes (lymphs) were found in obese vs. lean cats.

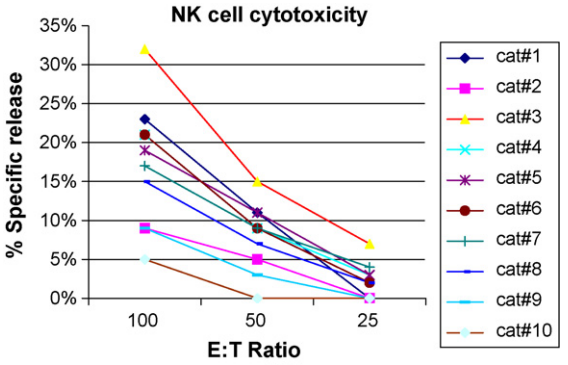

Fig. 1. Natural killer cell cytotoxicity in lean (#8 and 10) and obese cats (#2, 5, and 7) fed a diet containing polyunsaturated fatty acids, and lean (#9) and obese (#1, 3, 4, and 6) cats fed a diet containing saturated fatty acids. The results represent percent specific Cr 51 release of target cells from blood of 10 cats.

each population. Table 3 shows that no significant differences were found between the groups of cats in all the cell populations tested.

3.3. Lymphocyte function analysis

While the absolute numbers of the different subset of lymphocytes did not differ significantly between the different groups of cats, it was of interest to know whether differences in immune activity could be due to activation/inhibition of particular functions in subsets of cells. Lymphocytes were tested for natural cytotoxic activity and proliferation in response to a diverse panel of mitogens. Natural killer cell activity was tested against the cat feline cell line target cell FL74. Fig. 1 shows the results obtained in a typical day where 10 cats were tested at different E:T ratios. Cytotoxicity was dose dependent with a decrease at lower effector cell present in the assay. The results from all the groups of cats are presented in Table 4. Although the differences were not statistically significant, there appeared to be a higher cytotoxic activity in the obese B group of cats.

The proliferative capacity of lymphocytes from different groups of cats was next analyzed and the results are shown in Table 5. In this experiment, results were obtained from only 20 cats (6 lean and 14 obese) due to contamination of the 96-well plates. The results show that the functional activation of polyclonal stimulators of B cells (LPS), T cells (PHA, Con A), and lymphocytes in general (PMA/Ca ionophore) was not significantly different in lean and obese animals.

3.4. Phagocytic capacity of peripheral blood leukocytes

The ability of blood mononuclear cells was next investigated using fixed bacteria: a Gram positive (*S. aureus*) and a Gram negative (*E. coli*). Two different parameters were assessed in this assay. The percentage of positive cells is an indication of how many cells in the population were fluorescent for each bacterium. In addition, the peak channel number (PC#) is an indication of the fluorescence intensity, which represents the number of bacteria that the specific population took inside the cells. Both of these numbers are shown in Table 6. The results show that the function of blood phagocytic cells is not measurably different in the different populations of cats.

Table 3
Phenotyping analysis of peripheral blood of different lymphocyte subsets in two groups of 6 lean and 12 obese cats fed either a diet containing $n-3$ polyunsaturated fatty acids (3-PUFA) or saturated fatty acids (SFA)

Groups	B cells[a]	T cells[a]	CD4 + T cells[a]	CD8 + T cells[a]	NK cells[a]
Lean 3-PUFA	18.0 ± 5.2	25.3 ± 11.1	11.3 ± 4.2	9.8 ± 4.4	13.0 ± 3.2
Lean SFA	19.66 ± 3.5	25.33 ± 6.6	11.83 ± 2.5	11.50 ± 3.4	14.16 ± 4.1
Obese 3-PUFA	20.14 ± 4.8	22.00 ± 6.5	10.28 ± 2.6	10.50 ± 2.7	15.07 ± 3.6
Obese SFA	18.50 ± 2.2	28.21 ± 12.6	12.00 ± 4.3	11.14 ± 5.4	13.92 ± 2.9

[a] Numbers represent the percent of positive cells in the population of PBL with the defined fluorescent cell markers. Results confirm the visual examination of cells in that no significant differences were found. The numbers represent the percent of positive cells that were fluorescent with the cell marker in the PBL.

Table 4
Summary of cytotoxicity results from 12 lean and 24 obese adult neutered cats evenly allocated to two groups fed either a diet containing $n-3$ polyunsaturated fatty acids (3-PUFA) or saturated fatty acids (SFA)

GROUPS	100:1	50:1	25:1
Lean 3-PUFA ($n=6$)	13.3 ± 8.1	5.3 ± 4.3	2.5 ± 2.3
Lean SFA ($n=6$)	15.2 ± 4.1	6.7 ± 2.9	1.7 ± 1.8
Obese 3-PUFA ($n=12$)	15.2 ± 6.3	6.6 ± 3.9	2.3 ± 2.6
Obese SFA ($n=12$)	18.4 ± 8.5	9.4 ± 5.2	3.4 ± 3.4

The numbers represent percent specific Cr 51 released.

4. Discussion

Obesity has been characterized as a state of systemic low-grade inflammation suggesting a link between metabolism and immune function (Wang et al., 2004). In addition, obesity has been shown to predispose individuals to an increased risk of many diseases including atherosclerosis, diabetes, non-alcoholic fatty liver disease, certain cancers and asthma. Important pathways that link metabolism with the immune system and vice versa have been identified. Many of the interactions between the metabolic and immune systems are orchestrated by a complex network of soluble mediators of immune cells and adipocytes (Tilg and Moschen, 2006). Adipocytes are the source of various cytokines including adiponectin, leptin, resistin, and visfatin, which are thought to provide an important link between obesity and various disorders (La Cava and Matarese, 2004; Wellen and Hotamisligil, 2005). Adiponectin is likely an important player in affecting immune function as it has been shown to have an anti-inflammatory effect on endothelial cells though inhibition of TNF-alpha induced adhesion molecule expression (Ouchi et al., 2000). Additionally, adiponectin inhibits NF-κB activation in endothelial cells and interferes with macrophage functions (Ouchi et al., 2000; Yokota et al., 2000); treatment of cultured macrophages with adiponectin can also inhibit their phagocytic activity. Adiponectin also induces the production of anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist (IL-1RA) by human monocytes, macrophages and dendritic cells and suppresses the release of interferon gamma by LPS-stimulated macrophages (Wolf et al., 2004). These observations suggested a strong link between obesity and innate immunity. The presence of adiponectin in T cell proliferation assays resulted in decreased ability to induce an allogenic T cell response (Wolf et al., 2004). Another obesity related adaptive immune function that is mediated though adiponectin is the inhibition of B cell lymphopoiesis (Yokota et al., 2003).

This is the first comprehensive study to our knowledge examining the immune system of obese cats. Several parameters were measured to compare the innate and adaptive immune capabilities of the lean cats with those of obese cats. Any change in the immune status of an animal can be expected to reflect on the leukocyte profile. Therefore, the distribution of white

Table 5
Lymphocyte proliferation results from 6 lean and 14 obese adult neutered cats fed either a diet containing $n-3$ polyunsaturated fatty acids (3-PUFA) or saturated fatty acids (SFA)

Groups	Media	PMA/Ca ion	PHA	Con A	LPS
Lean 3-PUFA, $n=4$	36.6 ± 18.8	66.0 ± 42.7	65.0 ± 36.8	67.2 ± 35.7	68.9 + 39.4
Lean SFA, $n=2$	35.9 ± 16.8	70.4 ± 46.7	82.2 ± 34.1	60.6 ± 50 7	71.2 ± 41.9
Obese 3-PUFA, $n=9$	32.3 ± 16.6	62.4 ± 31.1	63.9 ± 33.3	71.2 ± 38.7	61.5 ± 33.0
Obese SFA, $n=5$	41.2 ± 14.3	70.7 ± 21.8	77.4 ± 27.5	80.9 ± 29.9	70.2 ± 28.1

Values are expressed as the OD reading at ~530 nm of average of three wells in a 96 well plate, incubated for 24 h at 37 C.

Table 6
Phagocytosis of *Escherichia coli and Staphylococcus aureus* by feline peripheral blood leukocytes in two groups of 6 lean and 12 obese cats fed either a diet containing 3-omega polyunsaturated fatty acids (3-PUFA) or saturated fatty acids (SFA)

Groups	*E. coli*		*S. aurues*	
	Percent positive	Peak channel #	Percent positive	Peak channel #
Lean 3-PUFA	69 ± 6	11.8 ± 4.5	61 ± 16	78.2 ± 36.1
Lean SFA	65 ± 7	9.9 ± 2.3	61 ± 17	83.0 ± 44.4
Obese 3-PUFA	65 ± 11	11.0 ± 4.7	58 ± 15	70.7 ± 26.8
Obese SFA	71 ± 6	12.8 ± 10.9	66 ± 14	81.9 ± 23.9

blood cells was examined by two separate methods in two different laboratories using different methods but the number of distinguishable white blood cells was not significantly different in the lean vs. the obese population of cats. This is in contrast to a recent study in people where a positive correlation was seen between body mass index (BMI) and total WBC, neutrophils and lymphocytes (Tilg and Moschen, 2006). Similarly, BMI, glucose intolerance, and insulin resistance were strongly and positively correlated with WBC in a large study of Chinese patients (Dixon and O'Brien, 2006). The obese cats of this study also showed glucose intolerance during an intravenous glucose tolerance test (Wilkins et al., 2004), however, this did not influence the WBC count indicating that there is no overall change in the phenotype of the immune system with the development of obesity in cats.

However, it is possible that the functions of these immune cells could be altered, leading to a compromised innate or adaptive immune function. The function of innate immune cells was determined in two separate assays: NK cell activity was measured by killing of target cells and phagocytosis was measured by uptake of fluorescence-labeled bacteria. No significant difference was seen between lean and obese cats. There was a trend in the obese SFA group towards higher cytotoxicity; however, the data could not be resolved statistically. NK cells are a key component of innate immunity. In the peripheral blood, they account for 5–20% of lymphocytes. They promote rapid lysis of target cells, and are a source of cytokines but can also be activated by cytokines (Chen et al., 2006; Moretta et al., 2002). In obese people, the numbers of NK cells have been found to be in the normal range (Nieman et al., 1999; Seaman, 2000; Yokota et al., 2003). However, inconsistent results have been reported regarding the effect of obesity and NK cell cytotoxic activity (Nieman et al., 1999; Santagostino et al., 1999; Seaman, 2000). Diet components such as carbohydrate or fat influence cytotoxicity. Carbohydrates have been reported to be correlated positively and dietary fat negatively with IL-2 dependent stimulation of NK cell activity (Moriguchi et al., 1995). In that study, the composition of the fat was not determined. However, polyunsaturated fatty acids, in particular the 3-PUFA have been documented to inhibit basal and stimulated NK cytotoxicity (Dovio et al., 2004; Thies et al., 2001; Yaqoob et al., 1994).

The function of the adaptive immune system was studied by measuring the ability of polyclonal activators to stimulate lymphocyte growth and proliferation. Several mitogens were used. The conclusions from these studies were that the proliferative responses to the mitogens tested were not significantly different in lean and obese cats. Little information is available about specific immune responses in obese people. Although, it has been reported that obesity induced decreases in both T-lymphocyte responses to concanavalin A and B-lymphocyte response to pokeweed mitogen (Yokota et al., 2000). Obesity suppressed lymphocyte functions, NK activity, and lymphocyte mitogenesis in a population of elderly men and women (Nieman et al., 1999). It was found that the decreased mitogen response in obesity was due to the decreased uptake of glucose, the major energy source for lymphocytes during proliferation (Moriguchi et al., 1998). Glucose uptake into immune cells is carried out by the glucose transporter 1, which is independent of insulin (Peters, 2006). In obese Zucker rats, altered mitogenesis was associated with the decreased expression of this transporter (Moriguchi et al., 1998).

Our study clearly shows that obese cats do not have alterations in any of the general immune function as indicated by our series of assessments of immune function. It needs to be noted, however, that these cats only had been obese for a short time and our results might therefore not be reflective of any alterations in chronically obese cats. One also has to consider that the use of outbred populations of animals for immune-related studies poses the problem of great variability between individual animals in many of the immune functions tested. The only way to obtain statistically valid data in such case, is to use a high enough number of animals to detect ''trends'', such as those published in humans from large center studies. In addition, the sensitivity of the tests may not be high enough to detect subtle differences in the immune response. These subtle differences may be important in conditions such as the stress of surgery or hospitalization (where increased incidence of disease is observed). Other factors not measured in the present study (such as lymphokine expression) that are pivotal in the regulation of the immune response may be affected by obesity. These parameters were beyond the scope of the present study and the effect of body composition these parameters warrants further investigation.

Significant advances have been made towards understanding the relation between the obesity, inflammation and immunity and many of the mediators that link these have been identified. However, much remains to be understood regarding the molecular mechanisms involved in these interactions as well as similar interactions in other animal models. A comparative approach to these problems will have a significant

impact on pharmacological interventions to ameliorate immune dysfunctions in obese animals.

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