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Adipocyte Size Distribution in ob/ob Mice during Preobese and Obese Phases of Development[1] (39572)

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Adipose cell size and numbers in some strains of genetically obese rodents, such as the ob/ob mouse, have been shown to be elevated during development as well as in adult life (1, 2). These reports usually present only average cell size of the adipocytes along with an estimate of the total cell numbers in the fat body investigated or in the whole animal. The average cell size may misrepresent the development of adipose tissue because possible differences in the frequency distribution of cell size are ignored. Joosten and van der Kroon (3) examined cell size distributions in the ob/ob mouse. These authors only reported the cell size frequency distribution for three individual animals. Their presentation of data without formulation of a composite representation of several animals of particular phenotypes does not lend itself to statistical analyses.

In the genetically obese mouse (C57B1/6Jobob), adipocyte hyperplasia continues beyond the termination date for adipocyte proliferation, which is reported as 40 days for normal mice and 60 days for obese animals (1). This obesity segregates as a recessive trait and cannot visually be detected prior to 4–5 weeks of age (1, 4). Most workers have concentrated their efforts on the developmental stages after the phenotypic expression of obesity, due to the lack of an identifying test during the preobese phase. Many of their observations may be secondary to the obesity itself. Recently it was found that oxygen consumption of young obese mice is considerably less than that of nonobese animals during the preobese phase of development (prior to 4–5 weeks of age) and can be used as a simple test for differentiation between future obese and nonobese animals (4, 5). Therefore, it is now possible to examine parameters of the obese state during the preobese phase of development.

In the present investigation, the frequency distributions of adipocyte cell size in the small- and moderate-size ranges are examined. The possibility is explored that differences in adipose cell size frequency distributions present during the preobese phase of development may be used to discriminate between future obese and nonobese animals. A method is also suggested for collection of individual cell size frequency distributions, with uneven numbers of total cells, into groups suitable for statistical comparisons.

Materials and methods. All animals used in the study were selected from the obese (C57B1/6Jobob) mouse colony at Michigan State University. The colony was originally derived from heterozygote (ob/+) breeding pairs that were purchased from the Jackson Laboratory at Bar Harbor, Me. The animals were raised in a temperature-controlled room at 25–27°, and had free access to food (Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.) and water. The distribution of adipose cell size was studied during the preobese (less than 4 weeks of age) and obese (greater than 4 weeks of age) phases of development in ob/ob and non-ob/ob littermates. The ob/ob and non-ob/ob mice were identified at 3 and 4 weeks by the procedure of Kaplan and Leveille (4, 5), which uses a low oxygen consumption as a genetic marker for identification of animals genetically destined to become obese. Older animals could be visually distinguished as

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either ob/ob or non-ob/ob.

The mice were killed by decapitation at 3, 4, 6–8, 10–12, and 16–18 weeks of age. After exsanguination, the epididymal fat pads of males and the parametrial fat bodies of females were dissected free. Duplicate tissue samples were obtained from the older animals while only single tissue samples could be obtained from the younger mice. The tissues were immediately rinsed in 0.9% NaCl, gently blotted dry, weighed, and fixed in 3% OsO_4–50 m*M* collidine buffer, pH 7.4. The fixed adipose cells were subsequently isolated by serial filtration through sized nylon mesh by the method of Hirsch and Gallian (6). Adipose cell size distributions were obtained on each tissue sample with a Coulter electronic counter (Model B equipped with Model J size distribution plotter). A 400-μm aperature was used. The instrument was calibrated with corn pollen grains. Cells were counted at various settings to determine the settings above which electrical noise in the system was eliminated. Since we were interested in the development of smaller and moderate-sized cells, the diameter distribution between 40–92 μm was selected. The plotted frequencies of 25 categories on the distribution plotter were grouped into five size categories: 40–50, 51–60, 61–69, 70–80, and 81–92 μm.

The average cell size and cell size frequency distributions were calculated for individual animals. The average proportions for each size classification within a group were then determined as well as the average number of cells per sample that were counted. These proportions represent average-grouped data from four to six sample populations which contain an uneven number of total cells counted. This type of data does not lend itself to the use of the traditional chi-square to compare frequency distributions of one population with another. Therefore, a modified chi-square was performed. The average proportions for each size classification were multiplied by the average number of cells per sample to obtain an average cell distribution in each size category that conformed to the average-calculated proportions. These average numbers of cell distributions were subsequently used for performance of the chi-square test. Chi-square, analysis of variance, and discriminant analysis were performed at the Rutgers University Computer Center.

Results. The nonobese males exhibited an ordered distribution at 3 weeks of age (Fig. 1a). The largest proportion of cells was that with the smallest diameter counted, S_1, and the smallest proportion of cells was that with the largest diameter counted, S_5. Later in development, after 10–12 weeks, the order of the proportion of cells at each classification was reversed. The greatest proportion of cells was the largest cells counted, S_5, and the smallest proportion of cells was the smallest cells counted, S_1.

During the preobese phase of development, at 3 weeks of age, the ob/ob males (Fig. 1a) exhibited a frequency distribution different from that of nonobese mice. From high to low, the proportion of cells was that of size classifications S_4, followed by S_3, S_2, S_5, and S_1. This distribution had a somewhat high proportion of larger cells during the preobese phase. In many respects, the order of size classifications exhibited by ob/ob males during the preobese phase was similar to that observed in the later stages of development among the nonobese males. After obesity was visually present, the proportions of the size classifications at later stages were radically different from the distributions observed during the early stages of development in both the obese and nonobese males. The order of proportions from high to low, was S_5, S_1, S_4, S_2, and S_3 during the obese phase. This is a bimodal distribution, with high proportions of large and small cells, a pattern quite different from that of the nonobese animals.

The nonobese females (Fig. 1b) exhibited a frequency distribution of adipocytes during development that was similar to that of nonobese males (Fig. 1a). Again, from high to low, the order of proportions at 3 weeks was S_1, S_2, S_3, S_4, and S_5. The order was similar among the future obese females, but the proportions were significantly different from those of future nonobese animals (Fig. 1b, Table I). During the later stages, after 10–12 weeks, a bimodal frequency distribution of cell size was present among the obese females (Fig. 1b). High proportions of both large and small cells were present. As in the males, this bimodal distribution found

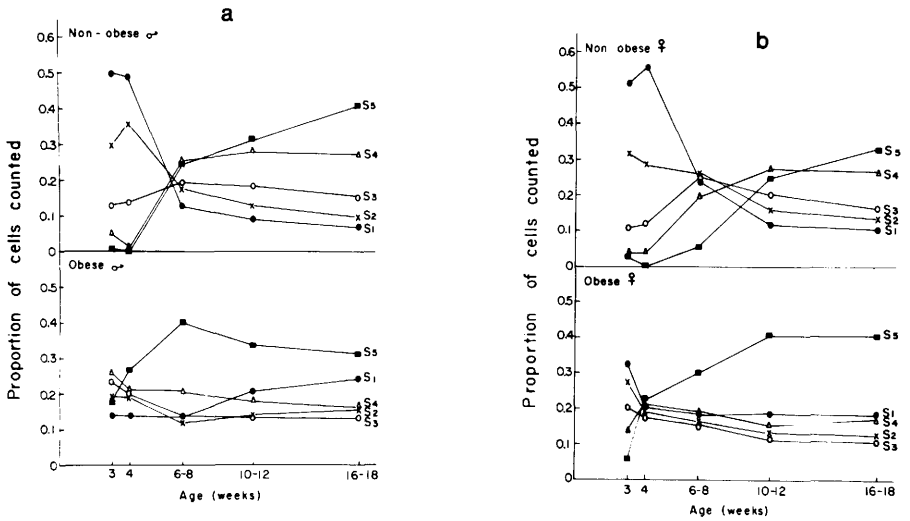

FIG. 1. Frequency of adipose cell size distribution during development in obese and nonobese mice. S_1 through S_5 refers to the diameter (micrometers) of cells in five size categories; S_1 (●——●) = 40–50 μm; S_2 (×——×) = 51–60 μm; S_3 (○——○) = 61–69 μm; S_4 (△——△) = 70–80 μm; S_5 (■——■) = 81–92 μm. Differences among the curves of obese and nonobese animals were evaluated by analysis of variance. In obese males, the curves of size classifications S_1, S_2, S_4, and S_5 were significantly different from nonobese males with respect to age, phenotype, and age-phenotypic interaction at $P < 0.001$. Among the females, the curves of size classifications S_1, S_2, S_3, S_4, and S_5 were significantly different from nonobese females with respect to age at $P < 0.001$ or $P < 0.025$. With respect to age-phenotypic interaction among the obese females, the curves of S_1, S_3, S_4, and S_5 were significantly different from the nonobese at $P < 0.025$ or $P < 0.001$.

among the obese females at 16–18 weeks was significantly different from the distribution among the nonobese females (Table I). A summary of the analysis of variance for two size classifications is presented in Table II. Only the smallest and largest sizes counted are presented as these are the most interesting. The analysis of variance indicates that the developmental patterns of both S_1 and S_5 class cell sizes are significantly different between obese and nonobese animals. It also indicates that the patterns significantly change throughout development. Both the chi-square test (Table I) and the analysis of variance (Table II) substantiate that the bimodal distributions observed among obese animals after 10 weeks of age are statistically significant. These bimodal distributions indicate that the number of small adipocytes has continued to increase in the obese animals throughout development as suspected by Johnson and Hirsch (1). Nonobese animals, in contrast, do not continue to proliferate adipocytes (1).

The bimodal frequency distribution of adipocytes among the obese animals would have been completely undetected if we examined only the average cell size (Table III) from the preobese through the obese phases of development. In this situation, the average cell size was misleading. The increased number of both large and small cells in the distribution (Fig. 1a) would have been missed if only average cell size were examined. Also, during the later stages of development, at 10–12 and 16–18 weeks, the average cell size calculated for the obese males was smaller than that for the nonobese males (Table III). Among the female animals, no differences in average cell size were found at these ages. The bimodal distributions found in both obese males and females (Fig. 1) help to explain these average cell size findings at 10–18 weeks of age (Table III). Reliance on average cell size at later stages missed that the majority of cells among the obese animals was larger cells (Fig. 1). Concomitant increases in the proportion of small cells resulted in a decreased average cell size (Fig. 1; Table III).

We were confronted with the problem of how to conduct a test on proportions in order to determine whether the population

TABLE I. AVERAGE ADIPOCYTE POPULATION DISTRIBUTION IN OBESE AND NONOBESE MICE.[a]

Age (weeks)	N	Phenotype	40-50 μm	51-60 μm	61-69 μm	70-80 μm	81-92 μm	Average number of cells per sample
			Preobese phase					
3	6	Obese ♂	1079	1474	1766	1952	1343	7564
	6	Nonobese ♂	2408	1440	641	238	21	4817
			$\chi^2 = 3160, P < 0.001$					
	5	Obese ♀	1442	1225	908	621	273	4462
	5	Nonobese ♀	1825	1124	383	133	110	3563
			$\chi^2 = 555, P < 0.001$					
4	6	Obese ♂	1055	1428	1464	1514	2004	7476
	4	Nonobese ♂	3777	2776	1087	87	0	7723
			$\chi^2 = 5294, P < 0.001$					
	4	Obese ♀	1305	1247	1134	1346	1431	6469
	4	Nonobese ♀	5443	2778	1152	369	0	9764
			$\chi^2 = 4634, P < 0.001$					
			Obese phase					
6-8	5	Obese ♂	249	214	244	372	722	1799
	4	Nonobese ♂	1050	1445	1631	2120	2018	8264
			$\chi^2 = 208, P < 0.001$					
	5	Obese ♀	441	387	372	460	711	2364
	5	Nonobese ♀	2026	2192	2108	1638	477	8426
			$\chi^2 = 1163, P < 0.001$					
10-12	4	Obese ♂	353	236	225	302	566	1680
	5	Nonobese ♂	827	1190	1683	2543	2824	9069
			$\chi^2 = 262, P < 0.001$					
	5	Obese ♀	418	296	253	343	899	2210
	6	Nonobese ♀	1328	1775	2265	3040	2776	11178
			$\chi^2 = 422, P < 0.001$					
16-18	4	Obese ♂	566	358	303	356	719	2300
	5	Nonobese ♂	588	839	1333	2333	3487	8577
			$\chi^2 = 743, P < 0.001$					
	5	Obese ♀	358	246	206	329	722	1910
	5	Nonobese ♀	752	958	1144	1872	2300	7028
			$\chi^2 = 188, P < 0.001$					

[a] At each size classification, the average percentage of cells in Fig. 1 was multiplied by the average number of cells counted per sample for the group to obtain the values in the table. These calculated values conform to the average frequency distribution of each group. Each group was compiled from samples which contained markedly unequal numbers of adipocytes.

distributions at any particular age were different in obese and nonobese animals. The number of cells counted in each sample was very variable. The average number of cells within a size classification obtained from four to six samples did not represent the average proportion of cells at that classification. This would be true only if the number of cells in each sample were approximately the same. Therefore, to help reduce this problem, we multiplied the average proportion of the samples at the particular size classification in question by the average number of total cells counted per sample to obtain the calculated average population distributions (Table I). From these numbers, a traditional chi-square was performed.

At every age studied, including the preobese phase of development, the adipocyte cell size distributions of the ob/ob mice were significantly different from the distri-

TABLE II. SUMMARY OF ANALYSIS OF VARIANCE FOR PROPORTIONS OF CELLS COUNTED AT TWO SIZE CLASSIFICATIONS.

Source	*df*	Mean squares	*F*	*P* > *F*
Males, Size 1				
Age	4	0.08110	16.60	0.001
Phenotype	1	0.10859	22.23	0.001
Age × phenotype	4	0.16870	34.54	0.001
Animal (age × phenotype)	41	0.00488		
Males, Size 5				
Age	4	0.15046	17.54	0.001
Phenotype	1	0.15024	17.52	0.001
Age × phenotype	4	0.04931	5.75	0.001
Animal (age × phenotype)	41	0.00857		
Females, Size 1				
Age	4	0.16046	23.32	0.001
Phenotype	1	0.07014	9.76	0.005
Age × phenotype	4	0.07730	10.75	0.001
Animal (age × phenotype)	39	0.00719		
Females, Size 5				
Age	4	0.18343	32.23	0.001
Phenotype	1	0.25138	44.18	0.001
Age × phenotype	4	0.02032	3.57	0.025
Animal (age × phenotype)	39	0.00569		

butions of the nonobese animals (Table I). The intraanimal variability was also fairly uniform throughout development for all size classifications studied (Table IV). Since the distributions at 3 weeks of age were extremely different for the future obese and future nonobese animals (Fig. 1; Table I), we hypothesized that the adipocyte cell size population distribution may be another useful way in which to discriminate between ob/ob and non-ob/ob animals during the preobese phase of development. To examine this hypothesis, a discriminant analysis (7) was performed with the aid of the Rutgers University computer. To discriminate between the ob/ob and non-ob/ob animals, the cell size distributions from the 3-week-old male and female mice were used. The resulting discriminant functions are shown in Table V. The discriminant function uses only four of the five adipocyte size classifications since the remaining fifth size classification is defined as a function of the remaining four. These discriminant functions (Table V) were then applied to each of the original animals at 3 weeks of age. The probability that each animal could be classified as either obese or nonobese was also calculated. All animals, but two obese females were correctly classified. The discriminant analysis suggests that the use of adipose cell size distribution may be another useful tool for differentiation of future obese and nonobese animals during the early preobese phase of development.

Discussion. Data were presented as composites of adipose cell size frequency distributions that represented four to six animals in a group. We also showed how such data could be manipulated so that they would be amenable to statistical analyses, such as a chi-square. Joosten and van der Kroon (3), in contrast, present adipose cell size frequency distributions for only three animals, with a statement that these are representative of the three genotypes, ob/ob, ob/+, and +/+. During the preobese phase of development, ob/ob and non-ob/ob individuals can be distinguished from one another on the basis of a lower oxygen consumption (5) as early as Day 17. It has also been suggested that ob/+ and +/+ individuals may also be distinguished on the basis of oxygen consumption values (5). Therefore, we would very much like to believe Joosten and van der Kroon (3) that these genotypes can be distinguished during the preobese phase on the basis of the adipocyte size frequency distributions. However, their presentation of data does not lend itself to statistical comparisons, such as chi-square or discriminant analysis. We also observed a sexual dimorphism in the frequency distributions at 3 weeks of age, which was not noted by Joosten and van der Kroon.

Johnson and Hirsch (1) observed that the total number of adipocytes increased until Day 60 in the ob/ob mice and to Day 40 in the non-ob/ob animals. They made the suggestion that this was due to an increase in the number of small adipocytes among the obese. Our data confirm this (Fig. 1). There was an increase in the frequency of small adipocytes throughout development which resulted in a bimodal frequency distribution

TABLE III. AVERAGE ADIPOSE CELL SIZE DURING DEVELOPMENT OF OBESE AND NONOBESE MICE.[a]

Age (weeks)	Obese ♂	Nonobese ♂	Obese ♀	Nonobese ♀
		Preobese phase		
3	67.4 ± 1.0 (6)	51.8 ± 1.8[b] (6)	58.6 ± 2.0 (5)	52.1 ± 2.0[d] (5)
4	68.7 ± 2.6 (6)	51.2 ± 1.0[b] (4)	66.4 ± 1.4 (4)	50.6 ± 1.1[b] (4)
		Obese phase		
6–8	72.4 ± 0.4 (5)	67.8 ± 2.6 (4)	68.6 ± 0.6 (5)	61.1 ± 2.3[c] (5)
10–12	68.9 ± 0.9 (4)	72.3 ± 0.7[c] (5)	70.6 ± 1.1 (5)	69.9 ± 1.6 (6)
16–18	68.0 ± 1.6 (4)	75.1 ± 0.9[c] (5)	70.8 ± 1.1 (5)	72.1 ± 2.1 (5)

[a] Values shown are the means ± SE with the number of observations shown in parentheses. The average cell diameter was calculated as a grouped mean from the frequency distribution of each sample over the range 40–92 μm. Preobese and obese phases refer to the developmental stages of genetically obese mice. Nonobese animals are matched by age to the obese animals.

[b, c, d] $F > P$ at 0.001, 0.025, and 0.05 levels with respect to the appropriate obese group.

TABLE IV. INTRAANIMAL STANDARD DEVIATION.[a]

	S_1	S_2	S_3	S_4	S_5	df
Males	0.0699	0.0515	0.0517	0.0373	0.0926	41
Females	0.0848	0.0533	0.0455	0.0473	0.0754	39

[a] The intraanimal standard deviations at each size classification are derived from the mean squares obtained in the analysis of variance. The units are proportions as in Fig. 1 and Table II.

TABLE V. DISCRIMINANT FUNCTION COEFFICIENTS.[a]

Adipocyte size class	Coefficient	Obese	Nonobese
	Female		
1	b_{i1}	447.83	457.87
2	b_{i2}	638.75	660.09
4	b_{i4}	920.59	910.17
5	b_{i5}	267.72	288.01
Constant	b_{i0}	−233.03	−243.53
	Male		
1	b_{i1}	728.93	742.08
2	b_{i2}	1724.83	1826.80
4	b_{i4}	1297.00	1238.33
5	b_{i5}	942.69	940.05
Constant	b_{i0}	−471.69	−491.84

[a] The adipocyte size classifications have been defined in Fig. 1 and in the text. The discriminant function is defined as $y_i = b_{i0} + b_{i1}S_1 + b_{i2}S_2 + b_{i4}S_4 + b_{i5}S_5$, where S is the proportion of adipocytes at the five size classifications as defined in Fig. 1 and in the text, and i = the phenotypic classifications; $i = 1$ or 2 for obese and nonobese, respectively. The probability that any animal can be classified as either obese ($i = 1$) or nonobese ($i = 2$) is defined as $p_i = e^{yi}/(e^{y1} + e^{y2})$.

only among the obese group. Bergen *et al.* (8) observed that the total protein content of hind legs in ob/ob males was similar to that of non-ob/ob animals until Week 6. After Week 6, the total protein content of the hind legs was considerably lower in the ob/ob than in the non-ob/ob animals. This approximates the date suggested by Johnson and Hirsch (1) for termination of adipocyte proliferation in the non-ob/ob animals. In the present investigation, we observed that the frequency of small adipocytes in the ob/ob males continued to increase after Week 6 (Fig. 1a). It can be hypothesized that adipocyte proliferation or recruitment occurred at the expense of lean or muscle growth in the leg preparation. Greenwood and Hirsch (9) suggest that preadipocytes may be formed until Day 35 in rats. Factors which regulate the differentiation of blast cells into preadipocytes or premuscle cells in the early neonatal period may be altered in ob/ob animals. This hypothesis has yet to be tested.

The adipocyte size frequency distributions were noted to be statistically different among ob/ob and non-ob/ob animals during the preobese phase of development. The results of the discriminant analysis suggest that the adipocyte size frequency distribution may be used for the early identification of ob/ob and non-ob/ob animals. A possible application to large domestic animals and

man may be made. The ob/ob and non-ob/ob animals were identified by the screening method of Kaplan and Leveille (4, 5), which uses the lower oxygen consumption as the identifying trait. The application of oxygen consumption as a screening device in large domestic animals and human children would be very cumbersome and expensive. Utilization of adipocyte size frequency distributions as a tool for early identification of potentially obese farm animals and children would be much easier. It would also not be necessary to determine the total body fat content to estimate the total adipocyte numbers for the animal. Only the adipocyte size frequency distribution would be necessary if it can be statistically compared by discriminant analysis to the frequency distributions obtained from known genotypes. This would be less expensive and faster than the determination of oxygen consumption in large animal chambers. Potentially obese meat animals could then be eliminated without wasting valuable feed grains on their growth. Potentially obese children could be carefully followed medically and guarded by parents to prevent a devastating childhood onset obesity which is difficult to treat.

Summary. The adipocyte size frequency distributions were determined during the preobese and obese phases of development in ob/ob and non-ob/ob mice. Only the small- and moderate-sized cells were evaluated. A procedure was presented that transformed composite data from the population distributions of individual samples into a form suitable for comparisons by chi-square. Among the obese animals, the frequency of small cells continued to increase during development, which resulted in a bimodal distribution after 10 weeks of age. During the preobese phase of development at Week 3, the adipocyte size frequency distribution of the ob/ob mice was significantly different from that of non-ob/ob animals. Discriminant analysis suggested that adipocyte size frequency distributions may be used to identify ob/ob animals during the preobese phase of development.

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