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Establishment of preadipocyte clonal line from epididymal fat pad of *ob/ob* mouse that responds to insulin and to lipolytic hormones

(adipose conversion/lipoprotein lipase/lipogenic enzymes)

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ABSTRACT **A clonal cell line that responds to insulin and to lipolytic hormones has been established from the epididymal fat pad of the C57BL/6J *ob/ob* mouse. This line, designated ob 17, has a doubling time of 12.5 or 19 hr in 10% or 1% fetal calf serum, respectively. It presents a heterogeneous chromosome number with 40% of the cells containing 35–44 chromosomes and expresses the characteristic H2-LA antigen. After cessation of growth, ob 17 cells accumulate droplets of triglycerides; this accumulation occurs to a significant extent even in the *absence* of insulin normally added after confluence. Lipoprotein lipase activity is negligible in exponentially growing cells but appears at its maximal level just after confluence with or without insulin. Acid:CoA ligase and acylCoA:diglyceride acyltransferase develop later than lipoprotein lipase. The appearance of lipolytic and lipogenic enzymes, but not of triglycerides, seems to be independent of the presence of lipoproteins or of unesterified fatty acids in the culture medium. Therefore, the differentiation program becomes operative when growth is arrested, and differentiation occurs, providing a source of exogenous lipids. Differentiated ob 17 cells in which endogenous triglycerides have been prelabeled on the fatty acid moiety do respond to epinephrine and corticotropin by release of radioactive fatty acid. This lipolytic response is counteracted by prior addition of insulin. The ob 17 cell line appears to be a useful model for study of growth and differentiation of adipose cells as compared to preadipocyte cell lines from the nongenetically obese mouse.**

Green and his associates (1–4) have reported the isolation of clones from the original stock of Swiss 3T3-M fibroblasts that acquire at the resting state the morphology of adipocytes under defined culture conditions. The transition to adipocyte is accompanied by (*i*) a coordinate induction of acetyl-CoA carboxylase, pyruvate carboxylase, fatty acid synthetase, and ATP-citrate lyase (5, 6), (*ii*) the acquisition of responsiveness to corticotropin, an increased response to β-adrenergics, and an increase in insulin receptor levels (7, 8), and (*iii*) the net induction of the triglyceride pathway enzymes and of lipolytic enzymes including lipoprotein lipase (9–13). The preadipocyte cell lines so far studied (3T3-L1 and 3T3-F442A) are interesting models for learning more about the factors controlling the multiplication and the enlargement of adipose cells, both of which occur *in vivo* and, when perturbed, lead to obesity in mammals (14). However, because the established 3T3-M cell line was isolated from disaggregated Swiss mouse embryo, we decided to start the isolation of preadipocyte cell lines from a known cellular origin.

The present communication describes the establishment and some properties of a clonal line of adipose cells from genetically obese C57BL/6J *ob/ob* mice. The ob 17 cells acquire, at the resting state, the key characteristics of adipocytes—that is, triglyceride accumulation linked to the appearance of lipolytic and lipogenic enzymes as well as a hormonal response to lipolytic agents and to insulin.

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MATERIALS AND METHODS

Culture Methods and Media. Epididymal fat cells were isolated from 8-week-old *ob/ob* mice according to Rodbell (15). The adipocyte fraction was thoroughly washed under sterile conditions and was free of any visible stroma or vascular cells. As described by Poznanski *et al.* (16), 10^4–10^5 fat cells were "sandwiched" between two coverslips (15-mm diameter) and allowed to dry for 15 min in a culture dish (35-mm diameter). Then, 1.5 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 200 units of penicillin, 50 μg of streptomycin, and 10 μg of tetracycline per ml was added and the dish was incubated at 37°C in a humidified 5% CO_2 atmosphere. The fat cells were fed every other day with the same medium for 12 days, during which all visible fat droplets disappeared and the cells became fibroblast-like. In contrast to the cells of the stroma/vascular fraction grown in parallel, no multiplication of the fat cells could be observed during the "dedifferentiation" phase. The dedifferentiated cells were trypsinized [0.25% trypsin in Ca^{2+}- and Mg^{2+}-free pH 7.4 phosphate-buffered saline (P_i/NaCl)], centrifuged, and inoculated with fresh culture medium (500–5000 cells per 60-mm-diameter culture dish). The cells were fed twice a week. By 8–10 weeks later, one colony had appeared. This colony was subcloned and the ob 17 clone was selected from among the 15 subclones on the bases of good contact inhibition and a high frequency of differentiation to adipose cells at the resting state. All experiments were performed with cells obtained after at least the 10th passage, corresponding to approximately 60 generations under our conditions, although at least 35 passages (150–200 generations) have been performed since then with no detectable change in the growth rate and in the adipose conversion. Unless otherwise stated, the adipose conversion was performed in the presence of insulin added after confluence (ob 17 cells could also differentiate in the absence of added insulin but at a slower rate).

Characterization of Cells. Measurement of labeled acetate and palmitate incorporation into lipids was performed in 1.5 ml of culture medium (35-mm culture dish) containing 0.5 μCi of [2-^{14}C]acetate or 0.25 μCi of [*U*-^{14}C]palmitate. Each dish was incubated for 15 hr (3 hr for palmitate) at 37°C in a humidified 5% CO_2 atmosphere. After removal of the medium, the cells were washed with P_i/NaCl, and the lipids were extracted and separated by thin-layer chromatography (9).

All other biochemical determinations and enzyme assays have been described (9). The method used for serum and bovine serum albumin delipidation were those of Cham and Knowles (17) and Chen (18), respectively. Lipoprotein-deficient serum (LPDS) was obtained by flotation of fetal calf serum at 16°C

Abbreviations: P_i/NaCl, phosphate-buffered saline, pH 7.4; LPDS, lipoprotein-deficient serum; LPLase, lipoprotein lipase; LDH, lactate dehydrogenase; DGTA, acylCoA:diglyceride acyltransferase or diglyceride acyltransferase.

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(64,000 × *g* for 44 hr) using KBr for direct adjustment of the density to 1.21 g/cm^3. The lipoprotein fractions, LPDS, and delipidized serum were dialyzed extensively against Pi/NaCl and sterilized by Millipore filtration. Karyotype analyses were performed on chromosomes prepared as described by McDougall (19) from actively dividing cultures (24 hr after seeding) arrested in metaphase with Colcemid (0.2 μg/ml) and then banded according to Gallimore and Richardson (20). Immunofluorescent staining of the characteristic H2-LA antigen was carried out on exponentially growing cells according to Pouysségur and Yamada (21). Growth in agarose was performed according to Montagnier (22). Tumorigenic activity of the ob 17 cell line was checked by injecting 10^6 cells into C57BL/6J mice 8–10 weeks old.

Materials. Swiss and C57BL/6J *ob/ob* mice were obtained from the Centre d'Elevage d'Orleans-La-Source. Culture media and fetal calf serum were from GIBCO, trypsin from Institut Pasteur Production, and collagenase (grade I) from Worthington. [2-^{14}C]Malonyl-CoA was purchased from New England Nuclear, [2-^{14}C]acetate and [*U*-^{14}C]palmitate were from the Commissariat à l'Energie Atomique, and all other labeled compounds were from Amersham Centre. [^{3}H]Palmityl-CoA was synthesized (9) by using CoA from P-L laboratories. The different lipids were products of Nu-Check-Prep. All other compounds and hormones were from Sigma. Antiserum H-2^d anti-H2^b (B 10.D2 anti-C57BL/10) was a product of Searle, and fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin fraction was a product of Miles.

RESULTS

Growth Behavior. Exponentially growing ob 17 cells exhibited a fibroblastic appearance (Fig. 1*A*). When entering the resting state, the cells did not show any lipid droplet accumulation for the first several days. No colony formation could be observed (Fig. 1*D*). At 7–10 days after confluence, the cells had enlarged considerably and were round. Two weeks later, triglycerides had accumulated as lipid droplets (Fig. 1*B*; Table 1) which in a small proportion of cells (<20%) ultimately fused into a few large fat globules (Fig. 1*C*). Oil Red O staining indicated numerous fat-accumulating centers (Fig. 1*D*). As observed with 3T3-L1 cells (2), the presence of BrdUrd during the exponential phase prevented adipose conversion (Fig. 1*E*). The histocompatibility antigen characteristic of C57BL/6J mouse was expressed in ob 17 cells (Fig. 1*F*) but no immunofluorescence could be observed either with control nonimmune mouse serum (Fig. 1*G*) or with cells from an established preadipocyte cell line isolated from the epididymal fat pad of Swiss mouse (unpublished data). The doubling time was short (Fig. 2) and not severely affected by low serum concentrations. The saturation densities in 10% and 1% fetal calf serum were 85,000 and 12,500 cells/cm^2, respectively; in the latter case, confluent cells presented a very flattened appearance. When inoculated into agarose medium (5 × 10^3 cells per 60-mm dish), only 12% of the cells had a limited ability to divide (six to eight cells per colony) but all cells were able to differentiate into adipose cells (not shown). ob 17 cells did not show any tumorigenic property 17 weeks after subcutaneous injection. The karyotypic properties were assessed at about 70 generations after isolation. Among 36 metaphases counted, 14 metaphases contained 35–44 chromosomes and the rest contained 53–134 chromosomes (mode, 62; 15%).

Lipid Metabolism. Table 1 (experiment 1) shows that, in contrast to the polar lipid content, the triglyceride content had increased significantly 2 weeks after confluence and reached a 24-fold increase, compared to the exponential phase, 3 weeks after the resting state was entered. [^{14}C]Acetate incorporation into lipids was low, indicative of a low endogenous fatty acid biosynthesis. In contrast, [^{14}C]palmitate incorporation into esters was high and, in agreement with the results reported below (experiment 2) and with Fig. 1*E*, the shift of palmitate incorporation toward triglycerides was blocked by BrdUrd. Table 1 shows also (experiment 2) that the specific activities of two characteristic enzymes of the triglyceride pathway, acid:CoA ligase and acyl-CoA:diglyceride acyltransferase, increased slightly after confluence but 3 weeks later showed 32-fold and

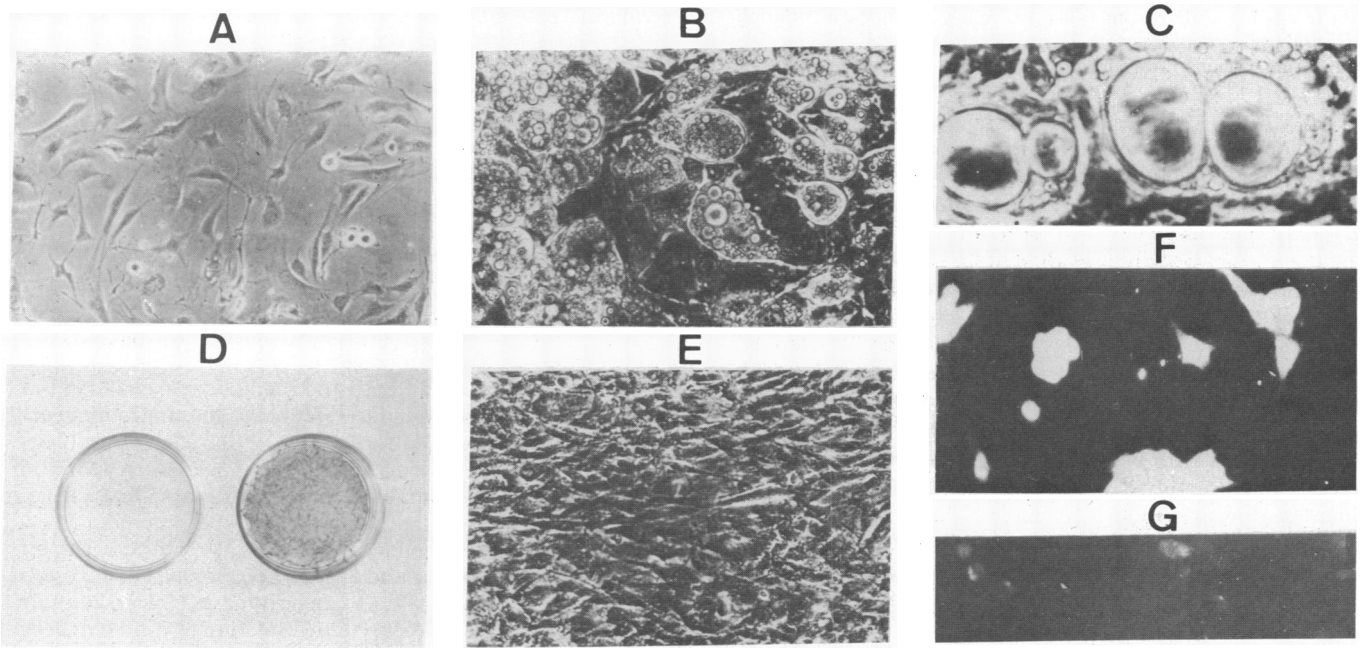

FIG. 1. (*A*) Exponentially growing ob 17 cells after 30 passages. (×400.) (*B*) Same cells 3 weeks after confluence (17 nM insulin). (×400.) (*C*) Same cells, showing large fat globules. (×600.) (*D*) Oil Red O staining of culture dishes 2 days (left) and 25 days (right) after confluence. (*E*) Resting cells treated with BrdUrd and deoxycytidine (as described in Table 1) 19 days after confluence. (×400.) Identical pictures were obtained with untreated cells 2 days after confluence. (*F*) Immunological characterization of H2-LA antigen on exponentially growing cells. (×400.) (*G*) Same cells treated with nonimmune serum. (×400.)

Table 1. Synthesis of lipids from [^{14}C]acetate and [^{14}C]palmitate, triglyceride content, and activity levels of LPLase and lipogenic enzymes during adipose conversion at various times relative to confluence

	−2 days	+2 days	+7 days	+14 days	+21 days	+21 days (+BrdUrd)
			Experiment 1			
Lipid content:						
Polar	3.0	5.0		5.5	1.8	
Neutral	2.0	1.8		24	47.4	
[^{14}C]Acetate → lipids (% of total added)						
Polar	0.85				1.1 (1.3)	
Neutral	0.74				0.72 (0.52)	
[^{14}C]Palmitate → lipids (% of total added)						
Polar	12	20			18.4	25.2
Neutral	23	22			31.0	15.0
			Experiment 2			
Acid:CoA ligase	0.15	0.7			5.4 (*20.0*)	0.25
AcylCoA:diglyceride acyltransferase	0.064	0.48			12.2 (*3.0*)	0.6
LPLase:						
Total	0.43 [0.38]	8.9 [4.8]			8.3 (*14.3*) [4.7]	
Released*	[0.2]	[3.36]			[2.36]	
Fatty acid synthetase	0.06				ND (*10.6*)	
LDH	2210	3600			3900 (*3700*)	
			Experiment 3			
LPLase:						
No insulin		11.4	12.4	4.0		
17 nM insulin		9.3		11.6		
17 nM insulin + 0.5 mM oleate			11.1			

Data of experiment 2 were obtained with ob 17 cells (20th passage) cultured in 5% fetal calf serum; 10% fetal calf serum was used in experiments 1 and 3 (35th and 20th passages, respectively). Unless otherwise stated, 170 nM insulin was added after confluence at every medium change. Where indicated, 10 μM BrdUrd and 30 μM deoxycytidine (2) were present during the exponential phase (approximately five generations) and during the resting phase. All enzyme activities are expressed in nmol of product formed per min per mg, except that the values in brackets are in nmol/min per dish (35 mm diameter) respectively for the total LPLase activity and that released. The values in parentheses (experiment 1) were obtained when the cells where maintained after confluence in the absence of insulin. The italic values in parentheses (experiment 2) were obtained with mature rat adipocytes. The lipid content (measured after separation by thin-layer chromatography) is expressed in neq of ester per μg of DNA (10^6 ob 17 cells correspond to 0.7 mg of protein and to 15 μg of DNA in exponentially growing cells, and to 0.9 mg of protein and 18 μg of DNA in resting cells). All values are the mean of duplicate (or triplicate) determinations on duplicate dishes. ND, not detectable. LPLase, lipoprotein lipase; LDH, lactate dehydrogenase.

* In serum-free medium containing 15 μM delipidized bovine serum albumin and 10 units of heparin per ml (1 hr at 37°C).

200-fold increases, respectively. As with 3T3-L1 fibroblasts, a net synthesis of these membraneous enzymes is likely to occur during differentiation (9). The presumed induction of the triglyceride-pathway enzymes was almost totally prevented by the presence of BrdUrd (experiment 2), which is in agreement with the observation that lipid droplets were absent (Fig. 1*E*). The low incorporation of [^{14}C]acetate into lipids was corroborated by the low levels of fatty acid synthetase, detected only during the exponential phase (experiment 2), and of ATP-citrate lyase (not shown) (no derepression of fatty acid synthetase was found with delipidized fetal calf serum or with medium supplemented with 0.25 μM biotin). Lactate dehydrogenase (LDH) not directly related to lipogenesis showed only a small increase during adipose conversion.

The specific activity of lipoprotein lipase (LPLase) was enhanced 20-fold within 2 days after the cells attained the confluent state and remained at this level thereafter in the presence of insulin; a large part of this activity was heparin-releasable (Table 1, experiment 2). The specific activity of LPLase in differentiated ob 17 cells was comparable to that obtained in mature adipocytes under the same assay conditions. After confluence, the LPLase activity reached similar levels with or without added insulin, but the presence of the hormone seemed to prevent the rapid decrease observed 1 week after confluence (Table 1, experiment 3). In the presence of insulin at 17 nM to 1.7 μM, the specific activity of LPLase measured 19 days after confluence was higher than when the cells were maintained in the absence of the hormone (Table 2). As observed for 3T3-L1 fibroblasts (9), the activity levels of acid:CoA ligase and of diglyceride acyltransferase were enhanced up to 10-fold and 4-fold, respectively, in the presence of insulin whereas LDH showed only a 1.6-fold increase.

The appearance of LPLase and the lipogenic enzymes did not seem to be substrate-induced (Table 3) and was reminiscent of the situation observed for LPLase with 3T3-L1 fibroblasts (12). In these experiments the different sera were added to confluent cells grown under normal conditions (10% fetal calf serum). It is apparent that the adipose conversion relies on exogenous lipids because endogenous fatty acid synthesis was low (Table 1, experiments 1 and 2). The need for exogenous esters through the action of LPLase can be bypassed by the addition of unesterified oleate to the culture medium of confluent cells. The addition of 0.1–0.5 mM oleate (as in Table 1, experiment 3) accelerated the rate of adipose conversion, causing a net triglyceride accumulation, and led to a complete conversion to adipose cells (not shown). The experiments reported in Table 3 indicate a lower percentage of adipose conversion than those

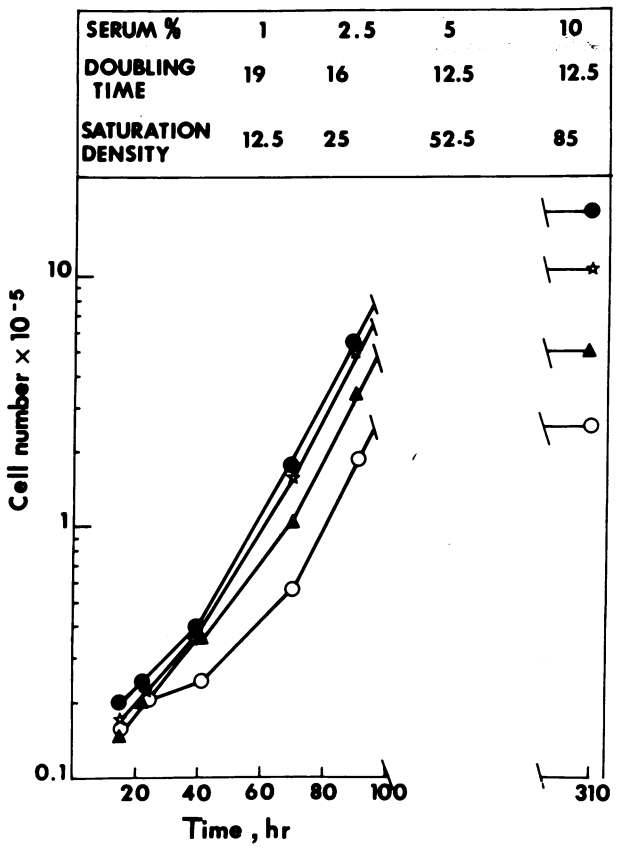

FIG. 2. Growth curves of ob 17 in media containing different amounts of fetal calf serum: ○, 1%; ▲, 2.5%; ☆, 5%; ●, 10%. Doubling times in hr and saturation densities in cells $\times 10^{-3}$ per cm^2 were determined with a Coulter Counter. Each reported value is the mean of duplicate dishes.

reported in Tables 1 and 2. It is possible that the extensive dialysis of the different sera before use caused a partial loss of some component(s) essential for differentiation.

Response to Insulin and to Lipolytic Hormones. Hormonal response was determined, on differentiated ob 17 cells prelabeled with [^{14}C]oleate, by measuring the radioactive fatty acid release (Fig. 3). Epinephrine (0.5 μM) or corticotropin (2 μM) produced a rapid and potent lipolytic effect. This effect was counteracted by 17 nM insulin (already visible at 0.3 nM), totally with epinephrine and partially with corticotropin. All of the released radioactivity cochromatographed with unesterified (free) fatty acids. The specific mobilization of radioactive endogenous triglycerides (above basal lipolysis) induced by 2 μM corticotropin was assessed by their net cellular decrease (16% after 3-hr incubation at 37°C) which was not observed with polar lipids.

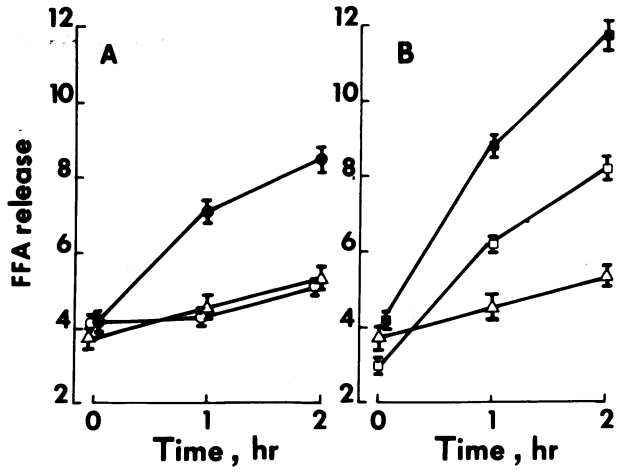

FIG. 3. Hormonal responses of ob 17 differentiated cells. Ob 17 cells were fed every other day for 14 days after confluence in medium supplemented with 10% fetal calf serum, 170 nM insulin, and 0.5 mM [^{14}C]oleate (67 dpm/nmol; 50,000 dpm per dish). No labeled oleate was added for the next 48 hr and no insulin was added for the next 24 hr. Each dish in triplicate (93,300 ± 5000 dpm per dish with 80% present in triglycerides) was incubated for 2 hr at 37°C in 2 ml of medium containing 1 mM bovine serum albumin with or without insulin. At time zero the different lypolytic hormones were added. (*A*) △, No addition; ●, 0.5 μM epinephrine; ○, 2-hr preincubation with 17 nM insulin, then 0.5 μM epinephrine. (*B*) △, no addition; ■, 2 μM corticotropin; □, 2-hr preincubation with 17 nM insulin, then 2 μM corticotropin. 0.2 ml aliquots were counted at times indicated. Total radioactivity (dpm per dish $\times 10^{-3}$) released from time zero is shown. Error bars are ±SEM. FFA, free fatty acid.

DISCUSSION

The established cell line from the epididymal fat pad of the *ob/ob* mouse exhibits a number of adipocyte properties. So far, ob 17 cells have undergone 180–200 generations (35 passages) and have shown no detectable change. The multiplication of ob 17 cells is rapid in 10% fetal calf serum and is slightly affected by low serum concentrations. Adipose conversion can occur significantly in the absence of added insulin. In contrast, an established preadipocyte cell line from Swiss mice (unpublished data) has a doubling time of 24 hr in 10% fetal calf serum and of 100 hr in 1% fetal calf serum; in contrast to the adipose conversion of the ob 17 clone and like that of the 3T3-L1 and 3T3-F442A clones (12, 13), the conversion was strictly dependent on added insulin. It is tempting to speculate that growth and differentiation of the "obese" and of the "normal" clones are somehow representative of the situation *in vivo:* there was an increase in the number of fat cells in genetically obese rodents compared to lean control animals, and this was not changed by underfeeding which corrects for the state of hyperinsulinemia (23).

The positive effect of increasing exogenous insulin on the

Table 2. Effect of insulin on adipose conversion and enzyme levels

Insulin added, nM	Conversion to adipocytes, %	LPLase	Acid:CoA ligase	DGTA	LDH
None	15–25	2.14	0.31	2.9	3900
17	35–45	6.8	1.03	7.2	6560
170	55–65	8.34	1.77	8.0	5410
1700	75–85	14.95	3.2	11.0	5770

After confluence, insulin was added every other day to resting cells (15th passage) during 19 days. All enzyme activities are expressed as in Table 1. The percentage of conversion corresponds to the lower and upper ranges of differentiated cells (containing visible lipid droplets) checked under the light microscope (×125) by two observers on (at least) 10 random fields.

Table 3. Development of LPLase and lipogenic enzymes in different media at different times (days) after confluence

	Serum additions											
	Untreated			Untreated + lipoproteins (×5)			LPDS			Delipidized		
	11	18	24	11	18	24	11	18	24	11	18	24
Acid:CoA ligase	0.38	0.47	—	0.41	0.89	—	0.24	0.89	—	0.51	0.79	—
DGTA	0.27	1.0	0.88	0.21	1.0	0.74	0.22	1.13	0.92	0.16	0.52	0.24
LPLase	2.8	1.9	4.94	1.94	2.32	3.46	0.89	3.0	5.71	2.3	—	5.9
LDH	3130	3385	3200	4040	3700	3900	3560	3715	3600	3140	3540	3400
Conversion to adipocytes, %	0–5	10–20	25–35	0–5	25–35	55–65	0–5	10–20	25–35	0	0	0–5

The different sera were added after confluence. All enzyme activities of confluent cells (26th passage) are expressed as in Table 1. As with other sera, the untreated fetal calf serum was extensively dialyzed against P_i/NaCl before use. The percentage of conversion was determined as in Table 2.

levels of LPLase, acid:CoA ligase, and diglyceride acyltransferase is reminiscent of the well-documented similar effect of insulin upon adipose tissue *in vivo* (24). The early and potent increase of LPLase activity in ob 17 cells just after confluence is similar to that reported in 3T3-L1 cells (12, 13). However, it occurs in ob 17 cells even when insulin is not added after confluence. Therefore, in every respect, confluent ob 17 cells just after cessation of growth behave as if they were either hyperresponsive to very low concentrations of insulin (or insulin-like activities) or might become independent of those factors during both cell multiplication and adipose conversion. However, the exposure to insulin seems necessary to sustain high levels of LPLase. The adipose conversion will occur if there is induction of the characteristic triglyceride-pathway enzymes and the addition of an exogenous source of lipids.

The low fatty acid biosynthesis observed in ob 17 cells differs from the higher but variable values reported for 3T3-L1 cells (3, 5, 9) and from the biotin dependency recently observed for triglyceride accumulation in 3T3-F442 A cells (13). The reasons for this low rate observed with ob 17 cells in culture remain to be determined; *in vivo*, the contribution of adipose tissue to total fatty acid synthesis becomes important in the *ob/ob* mouse (25). Although the induction of LPLase and of lipogenic enzymes does not seem to be substrate-induced, a possible induction by residual lipoprotein or apoproteins present in LPDS or by residual lipids (10% of the total esters) present in delipidized serum cannot be excluded. Most likely the differentiation program seems to be built-in in ob 17 cells and will allow triglyceride accumulation when being expressed after cessation of growth. The exquisite sensitivity of adipocytes to insulin and to lipolytic agents has been extensively shown (26). Under similar conditions, differentiated ob 17 cells are capable of responding, which indirectly suggests, as observed with adipocytes from the *ob/ob* mouse, the presence of the corresponding receptors and of the complete hormone transducing machinery including the hormone-sensitive lipase (27).

In summary, the ob 17 cell line from known cellular origin and isolated from the genetically obese mouse appears to be a useful system for study of the differentiation of adipose cells and also the growth factor requirements involved in cell multiplication. It should allow useful comparisons with the preadipocyte cell lines isolated from the nongenetically obese mouse.

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