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ARTICLES

Cytoplasmic Proteins of Porcine Adipocytes: Identification With Monoclonal Antibodies

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Abstract In the present study, monoclonal antibodies were produced using porcine adipocyte extracts as the immunogen. Two of the monoclonal antibodies, designated CB6 and IB4, exhibited reactivity toward only cells containing lipid in stromal-vascular cell cultures. The antigens recognized by the CB6 and IB4 monoclonal antibodies were 50 kD and 55 kD proteins, respectively. In vivo, IB4 immunoreactivity was detected only in lipid-containing cells, whereas immunofluorescence using CB6 was also detectable around muscle fiber bundles underlying the subcutaneous mesenchyme. In fetal subcutaneous mesenchyme, CB6 and IB4 immunoreactivities toward lipid-containing cells increased with developmental age, but each was not detectable in cells containing the smallest lipid droplets. In stromal-vascular cultures containing adipocytes, 48 hour treatment with the anti-lipogenic agent, growth hormone, only slightly altered CB6 immunoreactivity, whereas IB4 immunoreactivity was reduced by more than sixfold. The exact identity of the CB6 and IB4 antigens was not determined, but each may be useful as markers for studying regulation of adipocyte metabolism.

Key words: preadipocyte, growth hormone, immunocytochemistry

Enzymatic activities associated with lipogenesis and lipolysis, and lipid accumulation are indicators of adipocyte differentiation in vivo and in vitro. In recent years, a vast amount of information concerning adipocyte differentiation has been derived from studies of several preadipose cell lines, including certain subclones of 3T3 mouse embryo cells [1,2], 10T1/2 mouse embryo fibroblasts [3], and Ob17 epididymal fat pad cells [4]. It is generally accepted that conversion from preadipocyte to adipocyte is divided into early and late events in relation to expression of specific enzymatic markers [5,6]. In contrast to other developing systems, however, there are relatively few adipocyte-specific markers of differentiation, such as proteins identified by monoclonal antibodies. In addition, primary adipocytes and their precursors are seldom used to identify potentially important cellular markers. Changes in enzymatic activities which accompany adipocyte differentiation, as well as changes in cell morphology concomitant with lipid accumulation, should be accompanied by differential expression of a variety of interesting components. The objective of the present study was to identify cell markers expressed differentially during primary porcine adipocyte differentiation in vivo and in vitro using hybridoma technology.

MATERIALS AND METHODS

Monoclonal Antibody Production

One week-old crossbred pigs were the source of tissue for preparation of cell extracts for immunization and cell cultures for the initial hybridoma screen, respectively. Adipocytes were isolated by incubation of minced dorsal subcutaneous (sc) adipose tissue in a digestion buffer consisting of 0.1M Hepes, pH 7.4, containing 0.12M NaCl, 0.05M KCl, 0.001M $CaCl_2$, 0.005M glucose, 1.5% (w/v) bovine serum albumin, 1.6mg/ml collagenase (187U/mg, Cooper Biomedical) [7]. After a 60 min incubation at 37°C, a fivefold excess of digestion buffer was added and the suspension filtered through a 240μ nylon screen. Floating adipocytes were collected by centrifugation and washed twice using digestion buffer lacking enzymes. Fat cells were lysed in three volumes of ice-cold lysis buffer (10mM

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Tris-HCl, pH 7.4) by vortexing vigorously for 30 seconds. The suspension was centrifuged at 12,000 × g for 2 minutes, and the lipid-depleted cellular contents including pelleted material was stored at −70°C until further use. Protein concentrations were determined by the method of Lowry et al. [8].

The procedure used to prepare monoclonal antibodies (MAbs) has been described in detail by Kearney [9] and has been used to prepare monoclonal antibodies against adipocyte cell surface antigens [10]. 100 μg of cellular material was administered per mouse on each injection day. BALB/C mice were first injected with cellular material in complete Freund's adjuvant distributed subcutaneously into the rear footpads, inguinal, and axial regions. The injections were repeated three days later using incomplete Freund's adjuvant. Four more injections were given at three day intervals using saline alone. Twenty-four hours after the last injection, lymph nodes draining the sc areas of injection were used to prepare single cell suspensions. Lymph node cells were fused with SP2/0 myeloma cells using a 2:1 ratio of lymphocytes:myelomas and distributed into 24 well plates in RPMI 1640 medium (Sigma, St. Louis, Mo.) containing antibiotics, 20% heat inactivated fetal bovine serum, and HAT media supplements (10^{-4}M hypoxanthine, 4×10^{-7}M aminopterin, 10^{-5}M thymidine final concentration). Prior to screening, cultures were fed twice at 4 day intervals using the same medium containing hypoxanthine and thymidine but lacking aminopterin.

Cell Culture

Stromal-vascular (sv) cells from sc tissues were isolated as described under "Monoclonal Antibody Production." In order to rapidly produce sv cell cultures containing adipocytes for hybridoma screening, cells were seeded in 35 mm tissue culture dishes as spots (micromass culture) at a concentration of 10^6/ml using 0.035 ml of DME/F-12 medium containing 10% fetal bovine serum. Two mls of medium were added to the cultures after allowing 2 hours for cell attachment. Twenty-four hours after seeding, the medium was changed to DME/F-12 containing 2% pig serum for the remainder of culture. The high initial seeding density resulted in confluence at the onset of culture and rapid appearance of lipid-containing cells. For all other cultures, a subconfluent seeding density of 1×10^4 cells/cm^2 in 35 mm dishes was used. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 in DME/F12 medium supplemented with gentamicin sulfate (40 mg/L) and fungizone (2 mg/L). All cultures were initiated in medium containing 10% fetal bovine serum (FBS). After 3 days, cultures were maintained in medium containing 2% pig serum. In studies utilizing growth hormone, cultures were maintained in medium supplemented with 10% FBS for 3 days and then changed to medium supplemented with 2% pig serum for 5 days. Recombinant human growth hormone (Genentek) was reconstituted in sterile water and added (10nM final concentration) to serum-supplemented medium for the final 48 hours of culture.

Initial Screening

Supernatants from culture wells containing hybrid colonies were collected and tested for reactivity by immunofluorescence (see next section) using both intact and permeabilized micromass sv cell cultures. Positive hybrids were cloned twice by limiting dilution. Between each cloning round, wells containing hybrid colonies were tested for immunoreactivity by immunofluorescence. All hybrids in appropriately diluted test wells were positive after the second cloning round. Hybrid cells designated CB6 and IB4 were injected into pristane primed mice for ascites production.

Lipid-depleted cell and tissue extracts (50 μg/lane) were separated by electrophoresis on 7.5% polyacrylamide gels [11] and transferred to nitrocellulose sheets [12]. Sheets were treated with 1/500 dilutions of the appropriate ascites, or isotype-matched control antibody, followed by goat anti-mouse IgG (2×10^5 cpm/ml, 150 μCi/μg) iodinated by the chloramine T method [13]. Bands were localized by autoradiography. Results are representative of 5 separate experiments.

Immunofluorescence

Surface and cytoplasmic immunofluorescence was performed on sv cell cultures, cryostat sections of dorsal subcutaneous tissue from 50, 75, and 110 day fetuses, and cryostat sections of spleen, heart, liver, and kidney. All samples were tested a minimum of 4 times using fetal tissues from different litters. For surface staining, sv cultures and cryostat sections (24 μm) were rinsed twice with Hank's balanced salt solution (HBSS) containing 5% heat inactivated fetal bovine serum and incubated with the appro-

priate MAb (hybridoma supernatants during the initial screening and 1/500 diluted ascites during all subsequent procedures) for 20 min at 4°C, washed three times with HBSS, then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG at 0.1 mg/ml. For cytoplasmic staining, cultures and cryostat sections were fixed with 4% paraformaldehyde in PBS for 30 min at 4°C, then permeabilized with 0.1% Nonidet-P40 for 20 min and then stained as above. Cultures and sections were rinsed and mounted with Elvanol [14]. Oil red-O hematoxylin staining was performed as described by Hausman [15].

RESULTS

Hybrid Selection

Antibody-secreting hybrids of interest were detected by staining fresh and fixed/permeabilized sv cultures with supernatants from hybrid-containing wells, followed by immunofluorescent localization of bound antibody using FITC-conjugated goat anti-mouse IgG. Antibodies exhibiting reactivity toward both surface and cytoplasmic antigens were detected. Some of the wells contained antibodies which reacted with all cells in sv cultures, while others reacted with only a proportion of the cells. The hybridomas designated CB6 and IB4 stained only cells containing lipid in cultures, and therefore were chosen for further study. CB6 and IB4 were each cloned twice by limiting dilution and injected into pristane-primed mice for ascites production. Ascites from CB6 and IB4 each produced detectable cytoplasmic staining of adipocytes in cultures at dilutions to 1/20,000. Dilutions of 1/500 did not produce detectable background staining.

Antibody Specificity

As part of the initial characterization, CB6 and IB4 reactivity was examined using immunofluorescence on various tissues. Staining using either CB6 or IB4 was not detectable in sections of spleen, heart, liver, or kidney. However, immunofluorescence using the CB6 MAb was detectable around muscle fiber bundles in the muscle layer underlying the sc tissue as opposed to the cytoplasmic pattern detected in lipid-containing cells. Therefore, the possibility of non-specific binding of the CB6 MAb in vivo, or that the CB6 antigen is present in muscle, cannot be ruled out. On the other hand, IB4 immunoreactivity was not detected toward any cells in any non-adipose tissue.

SDS-polyacrylamide gel electrophoresis and blotting of adipose tissue extracts, and adipocyte cell extracts prepared in the same manner as extracts used for immunization, demonstrated different but closely migrating antigens for the CB6 and IB4 MAbs (Fig. 1). The CB6 antigen migrated with an apparent mw of 50 kD, whereas the IB4 antigen migrated as a larger protein of approximately 55 kD. The CB6 MAb produced

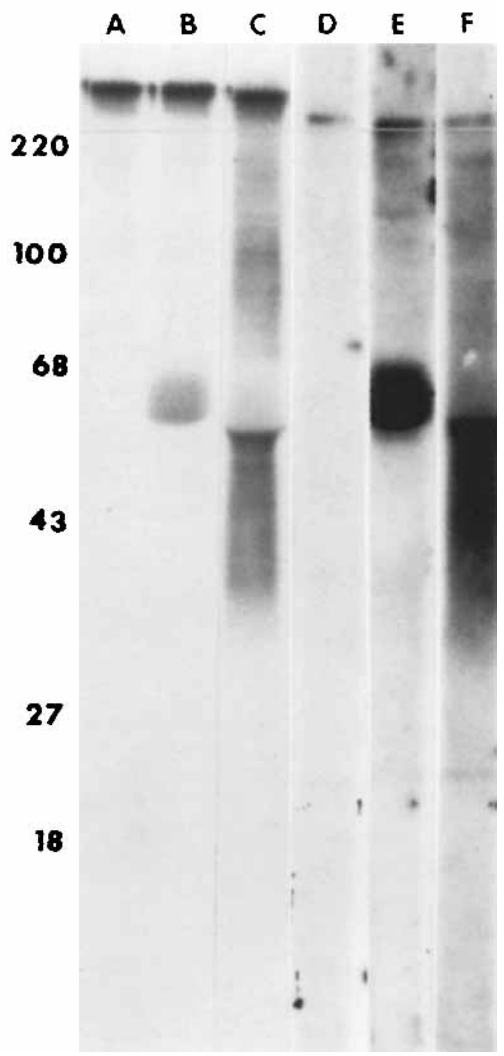

Fig. 1. Autoradiograph of proteins recognized by monoclonal antibodies. Samples were obtained either directly from homogenized subcutaneous adipose tissue or adipocytes isolated by collagenase digestion. Cells and tissues were homogenized and partially delipidated as described under "Materials and Methods." Proteins (50 μg per lane) were separated by SDS-polyacrylamide gel electrophoresis on 7.5% gels and blotted onto nitrocellulose. Extracted adipose tissue (lanes A–C) and extracted isolated adipocytes (lanes D–F) were screened with control MAb (lanes A,D), IB4 (lanes B,E) and CB6 (lanes C,F). Bound MAb was localized using ^{125}I-labeled goat anti-mouse IgG followed by autoradiography. The relative mobility of protein standards (in kilodaltons) is indicated on the left.

higher background on blots than observed using either IB4 or control MAb.

Monoclonal Antibody Reactivity In Vivo

Immunofluorescence was not detectable using either CB6 or IB4 in 50 day fetal sc tissues. At 75 days of gestation, CB6 and IB4 immunoreactivities were detected in lipid-containing cells (Fig. 2), but were not detectable in most cells containing the smallest lipid droplets. In 110 day sc tissues, the staining patterns for CB6 and IB4 were similar to sections stained with oil red-O (Fig. 3) in that each MAb stained all lipid-containing cells. However, adipocytes containing similar amounts of lipid exhibited different fluorescent intensities (Fig. 3).

Monoclonal Antibody Reactivity In Vitro

In vivo, CB6 and IB4 immunoreactivity was only detectable in tissues after the appearance of lipid. Immunoreactivity was then examined using sv cell cultures. Prior to the onset of lipid deposition (first three days on FBS), CB6 and IB4 reactivity was not detected except for the few cells containing lipid from the onset of culture. Replicate cultures containing cells differentiating in the presence of pig serum are shown in Figure 4. CB6 and IB4 each produced a diffuse pattern of cellular reactivity which was restricted to cells containing lipid droplets. In general, the intensity of fluorescence was related to the amount of lipid in the cells, with the brightest cells being those containing the most lipid. The CB6 MAb also produced a punctate staining pattern localized primarily in the perinuclear region. Cellular immunoreactivity was not detectable using control first antibody (Fig. 4) or if cells were not permeabilized (not shown).

The relationship between CB6 and IB4 immunoreactivity and lipid deposition was tested further by inhibiting lipid deposition with growth hormone and staining after 48 hours of treatment. In the absence of growth hormone, CB6

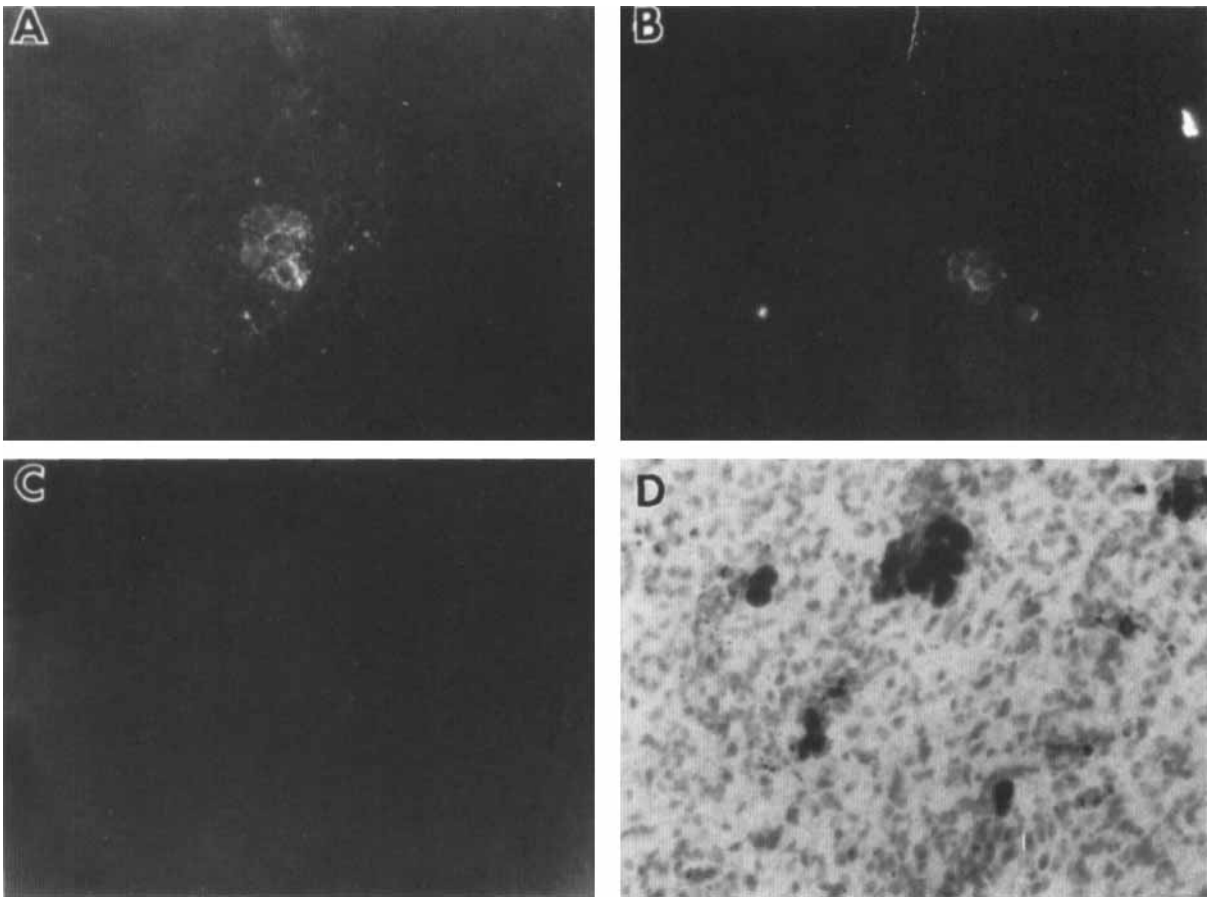

Fig. 2. Immunoreactivity of 75-day fetal subcutaneous adipose tissue toward the CB6 and IB4 MAbs. Replicate cryostat sections 24μ were fixed in 4% paraformaldehyde and permeabilized with 0.05% Nonidet P-40. Sections were stained with CB6 (**A**), IB4 (**B**), and control (**C**) MAbs, and with oil red-O/hematoxylin (**D**). Immunofluorescence was not intense in 75 day tissues, but was detectable specifically within lipid-filling cells. Bar = 50 μ.

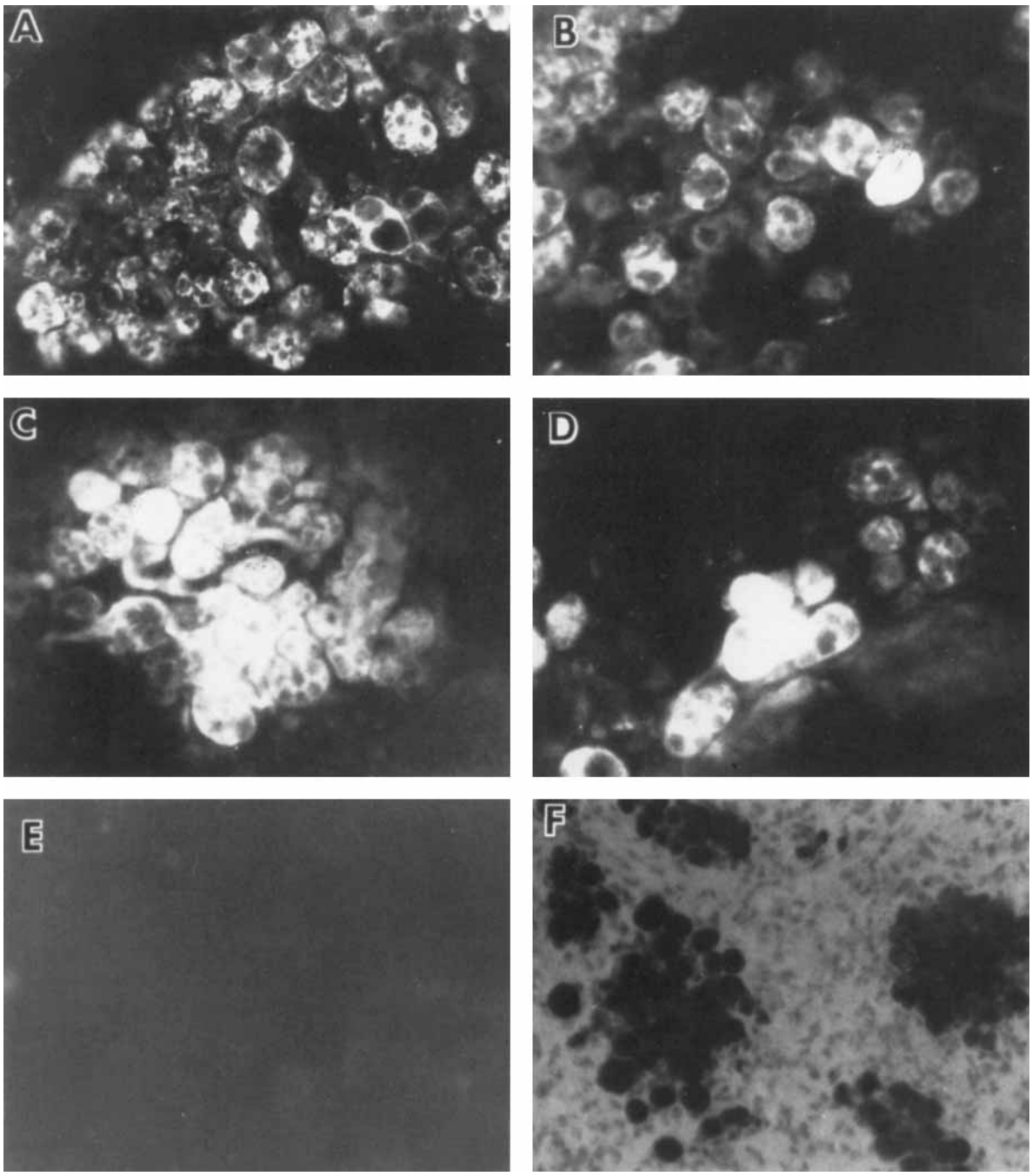

Fig. 3. Immunoreactivity of 110-day fetal subcutaneous adipose tissue toward the CB6 and IB4 MAbs. Replicate cryostat sections (24μ) were fixed in 4% paraformaldehyde and permeabilized with 0.05% Nonidet P-40. CB6 (**A,B**) and IB4 (**C,D**) produced cytoplasmic staining in all lipid-filled cells examined. Staining was not detectable using control MAb (**E**). Oil red-O/hematoxylin stained section (**F**) demonstrates lipid as well as cell density in sections. Cells lacking lipid in A–D are not detectable with either MAb.

and IB4 immunoreactivity was detectable in 93% and 85% of the cells containing detectable lipid, respectively (Table I). Treatment of cultures with growth hormone reduced fat cell cluster number to 20% of that in control cultures. The number of remaining lipid-containing cells with detectable levels of the CB6 antigen was only slightly diminished (Table I), whereas IB4 antigen expression by the remaining lipid-containing cells decreased from 85% to 13% in the presence of GH (Table I).

DISCUSSION

The primary objective of this study was to identify cytoplasmic antigens which would be markers for studying adipocyte differentiation

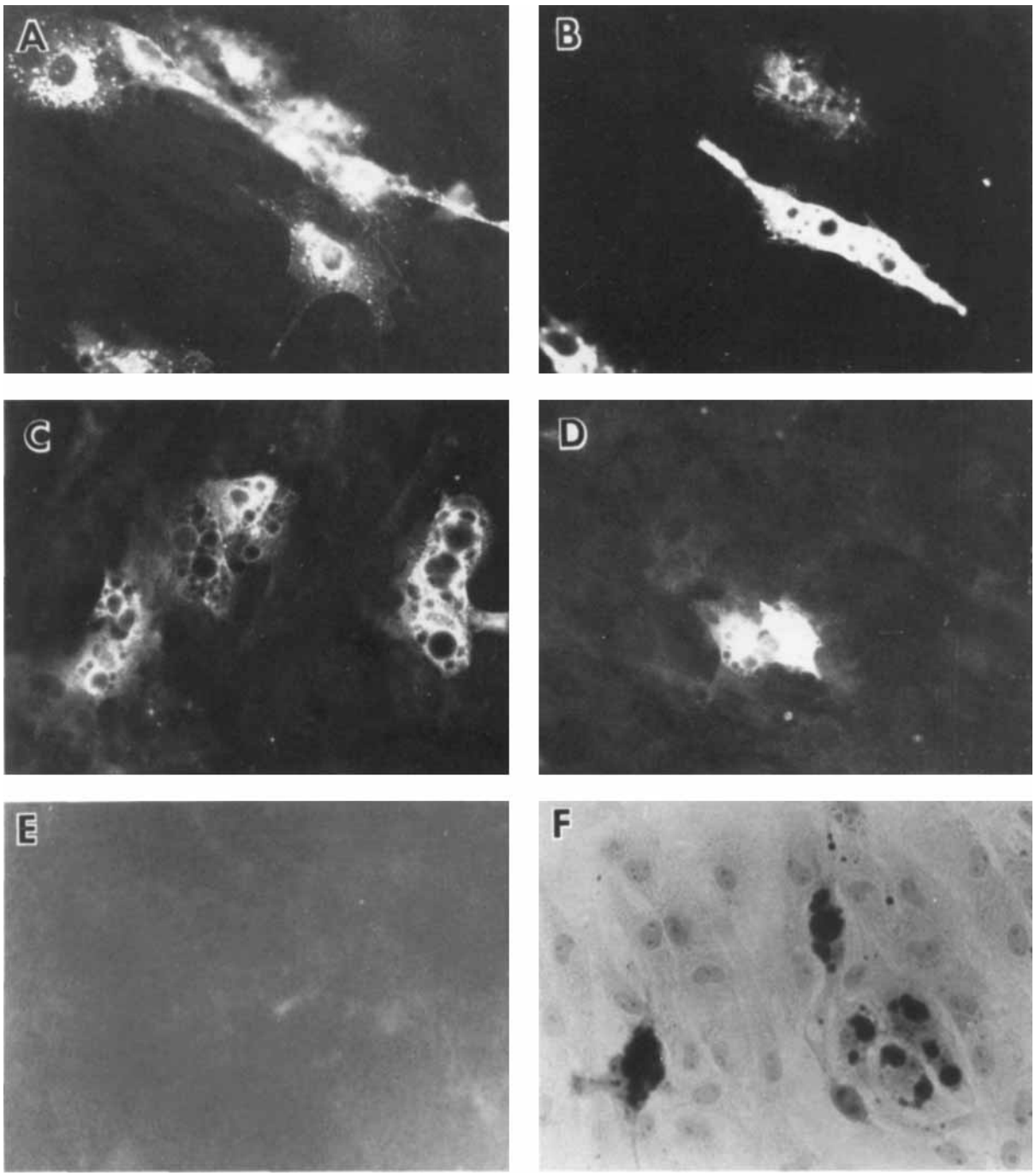

Fig. 4. Immunoreactivity of cultured stromal-vascular cells toward the CB6 and IB4 MAbs. Replicate cultures were fixed and permeabilized as described under "Materials and Methods." Immunofluorescence using CB6 (**A,B**), IB4 (**C,D**), and control (**E**) MAbs is shown. Immunoreactivity was detectable primarily toward cells containing lipid. Cells lacking lipid (shown in oil red-O/hematoxylin stained culture (**F**) are present in the fields shown in A–E but lack immunoreactivity. Some non-specific background fluorescence is shown in E. Bar = 50μ.

or regulation of lipid deposition. Monoclonal antibodies were produced which exhibit reactivity preferentially toward cells containing lipid in sv cultures. In cell cultures and sc adipose tissue depots, cytoplasmic lipid accumulation preceded detectable CB6 and IB4 immunoreactivity. Each antigen may have been present in preadipocytes prior to lipid deposition or even in cells completely lacking lipid, albeit at undetectable levels. In any case, the cells with the greatest quantity of each antigen were adipocytes. IB4 immunoreactivity was detectable solely in cells

TABLE I. Quantitation CB6 and IB4 Immunoreactive Cells and Fat Cell Clusters in Response to Growth Hormone Treatment

	Growth hormone	
Antibody[a,c]	−	+
CB6	93 ± 3	88 ± 3
IB4	85 ± 4	13 ± 2
Fat cell clusters[b,c]	8.1 ± 0.8	1.6 ± 0.1

[a]Percentage of cells (±SE) containing detectable lipid that also stain with the MAb.
[b]Clusters per mm^2 (±SE).
[c]Data represent counts obtained in 10 fields per tissue culture dish using 3 dishes per treatment in 4 separate experiments (n = 4).

containing lipid in vivo and in vitro, whereas the CB6 MAb also exhibited reactivity toward some structure in muscle beginning around 75 d of gestation. The presence of CB6 immunoreactivity in muscle was not investigated thoroughly in this study. However, it should be pointed out that lipid accumulation is detectable in muscle tissues [16,17].

In several experiments we were unable to accelerate the appearance of CB6 or IB4 immunoreactivity in vitro prior to lipid deposition. Since it was our interest to determine if CB6 or IB4 antigen expression is modulated in response to either lipogenic or lipolytic stimuli, the alternative approach was to treat cultures with compounds having a net negative influence on lipid deposition. Since growth hormone reduces lipid deposition in vivo [18] and lipogenic enzyme activities in sv cell cultures [19], immunoreactivity toward sv cells grown in medium supplemented with pig serum was compared to parallel cultures supplemented with pig serum and 10 nM growth hormone. After 3 days on FBS followed by 3 additional days on pig serum, cells containing lipid were clearly present in cultures (prior to treatment). Two days of subsequent growth hormone treatment only slightly altered CB6 immunoreactivity (Table I). Longer periods of treatment with growth hormone (> 96 hours) resulted in the appearance of a number of cells exhibiting CB6 immunoreactivity but lacking detectable lipid. Results using IB4 were strikingly different. In comparison, lipid-containing cells stained with the IB4 MAb were reduced more than sixfold in response to growth hormone, and the response was rapid in onset. Cessation of IB4 antigen expression may be an early event in the anti-lipogenic response to growth hormone, whereas CB6 antigen expression appears not to be effected early. The exact mechanism(s) regulating expression of these antigens in response to growth hormone are not known at this time. However, these antigens may be useful as markers for studying the regulation of lipogenesis and/or lipolysis.

Synthesis of many proteins is enhanced during adipocyte differentiation [20,21]. Enzymatic activities associated with lipogenesis and lipolysis, as well as extracellular and cytoskeletal components, exhibit differential expression during adipocyte differentiation (for review, see 22). Of the well characterized, differentiation-dependent polypeptides described for adipocytes, malic enzyme [21,23] and lipoprotein lipase (LPL, reviewed in 24) are both in the general molecular weight range as the CB6 and IB4 antigens. LPL immunoreactivity in situ in cultured Ob17 cells [5,25] exhibits a similar pattern of reactivity as the IB4 antigen. However, detailed descriptions of the location of other cytoplasmic adipocyte proteins are lacking. Furthermore, to the best of our knowledge there are no reports concerning in vivo distribution of cytoplasmic proteins, similar to CB6 and IB4, within adipose tissues. Therefore, it is impossible at this time to speculate further as to the identity of the CB6 and IB4 antigens, and whether their function has already been described. Further studies aimed at identifying the functionality of the CB6 and IB4 antigens together with the possible construction of molecular probes for these antigens would be in order.

In summary, this report describes immunoreactivity of murine monoclonal antibodies against cytoplasmic proteins found predominantly in adipocytes. A similar approach has resulted in identification of adipocyte cell surface antigens [10,26]. One of the MAbs, IB4, was detected solely in lipid-containing cells. Furthermore, the ontogenetic appearance of both CB6 and IB4 immunoreactivity within cells accumulating lipid in vivo and increased expression of each antigen by cells accumulating lipid in vitro indicated that the CB6 and IB4 antigens were expressed concomitant with lipid deposition. Expression of the IB4 antigen in particular was modulated in response to the anti-lipogenic agent, growth hormone. The identities and functions of the CB6 and IB4 antigens are not known at this time, and a molecular approach may well be required to identify each antigen with certainty.

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