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Cell Biology

Inability of serum from abetalipoproteinemic subjects to stimulate proliferation of human smooth muscle cells and dermal fibroblasts *in vitro*

(cell culture/cell proliferation/serum growth factors/growth control)

DON L. LAYMAN, BARBARA J. JELEN, AND D. ROGER ILLINGWORTH

Department of Medicine, Divisions of Cardiology and Metabolism-Nutrition, University of Oregon Health Sciences Center, Portland, Oregon 97201

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ABSTRACT **Serum from two patients with abetalipoproteinemia, a rare disorder of lipid metabolism characterized by the absence of chylomicrons and very low density and low density lipoproteins, did not stimulate the proliferation and growth of human smooth muscle cells or dermal fibroblasts *in vitro* as effectively as normal serum. The growth-promoting activity of this serum was comparable to that observed for lipoprotein-deficient plasma from normolipidemic subjects. Although the mitogenic effect of abetalipoproteinemic serum was improved with supplementation of low density lipoproteins, it was still about half the activity achieved with normal serum. However, the growth-promoting activity of this serum was completely restored to normal levels after addition of a lysate of normal platelets. In contrast, the mitogenic activity of lipoprotein-deficient plasma remained unchanged after addition of a lysate from abetalipoproteinemic platelets, whereas a similar supplementation of normal platelets completely restored its growth-promoting activity to normal. Thus, the inability of abetalipoproteinemic serum to promote growth appears to be due both to a deficiency of a platelet-releasable growth factor(s) and to the absence of serum lipoproteins.**

Nearly all cultured mammalian cells require serum in the culture medium for cell viability and proliferation (1–3). Serum contains a number of mitogenic substances, several of which, a platelet growth factor and plasma lipoproteins, stimulate the proliferation and growth of arterial smooth muscle cells and fibroblasts in culture (4–10).

The possible presence of mitogenic factors in platelets was first demonstrated by Balk (4), who observed that plasma had very little growth-promoting activity as compared to serum in stimulating the proliferation of cultured chicken fibroblasts. He postulated that the mitogenic factor(s) were released from precursors in plasma or from platelets when blood was clotted in the preparation of serum. Ross *et al.* (5) extended his observation by showing that monkey arterial smooth muscle cells grew poorly in the presence of monkey plasma in contrast to serum. They further demonstrated that the addition of platelets or a platelet-free supernatant to plasma fully restored its proliferative capacity to that of serum. Kohler and Lipton (6) obtained similar results with cultured 3T3 fibroblasts.

Of the serum lipoproteins that have been shown to enhance the proliferation of cultured cells, very low density (VLDL) and low density (LDL) lipoproteins are effective when added to medium containing lipoprotein-deficient plasma or growth-limiting concentrations of serum (7–9). High density lipoprotein exhibits minimal growth-stimulating activity. However, when VLDL and LDL were added to the culture medium in the absence of plasma or serum, they did not promote growth and were unable to maintain cells in a viable state in the resting phase (10). In several studies, VLDL and LDL isolated from both normo- and hyperlipidemic plasma were similarly effective in promoting cell proliferation (7, 8), whereas only LDL from hyperlipidemic plasma promoted cell growth in other studies (9). In view of the recognized association between premature atherosclerosis and certain hyperlipidemic states in which VLDL and LDL are elevated, studies were carried out to determine the growth of cultured human smooth muscle cells in the presence of serum from two patients with abetalipoproteinemia. This serum is devoid of chylomicrons, VLDL, and LDL and shows a complete absence of apoprotein B (11).

MATERIALS AND METHODS

Cell Culture. Cultures of human smooth muscle cells and dermal fibroblasts were established as described in detail elsewhere from tissue explants of aortic medial smooth muscle and skin from both a newborn infant and a 35-year-old man obtained at routine autopsy (12, 13). The tissue explants were cultured in McCoy's 5a medium (Flow) containing 20% pooled human serum, 200 units of penicillin per ml, and 200 μg of streptomycin per ml. Cells that grew out from the explants were harvested by trypsin/EDTA dissociation and then subcultured in medium containing 10% pooled human serum. Culture medium was changed twice weekly. To test the effect of abetalipoproteinemic serum (abeta serum) on cell proliferation, we dissociated smooth muscle cells and fibroblasts between the third and eighth passage (1:2 split) with 0.05% trypsin/EDTA solution, collected them by centrifugation, and resuspended them in McCoy's 5a medium containing the test or control serum. Approximately 2–2.5 $\times$ 10^5 cells were pipetted into 25-cm^2 tissue-culture flasks. At 1, 2, 3, 4, 7, and 10 days after seeding, the number of cells in duplicate or triplicate flasks was counted with a Neubauer hemocytometer.

Preparation of Human Serum and Serum Lipoproteins. Human serum, pooled from about 600 donors, was obtained from the Oregon Red Cross Bank (Portland, OR). It was sterilized by membrane filtration followed by heat inactivation at 56°C for 30 min. Serum cholesterol and triglycerides were analyzed by standard methods on a Technicon Autoanalyzer II (14). Serum cholesterol and triglyceride levels were 194 and 128 mg/dl for normal and 26 and 8 mg/dl for abetalipoproteinemic serum, respectively.

LDL, lipoprotein-deficient plasma of $\rho > 1.21$ g/ml, and a serum fraction of $\rho > 1.063$ g/ml were isolated by the ultracentrifugation flotation procedure of Havel *et al.* (15). Blood (50–100 cm^3) was obtained by venipuncture from two patients with abetalipoproteinemia after informed consent and in conformity with institutional policy. The blood was allowed to

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Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; abeta serum, abetalipoproteinemic serum.

clot; the resulting serum was separated by centrifugation, heat inactivated at 56°C for 30 min, and then sterilized by membrane filtration.

RESULTS

Response of Cultured Human Cells to Abetalipoproteinemic Serum. The ability of abeta serum to promote the proliferation and growth of cultured aortic smooth muscle cells or dermal fibroblasts was compared to that of lipoprotein-deficient plasma and whole human serum. Fig. 1 shows the change in cell number with time in cultures of adult and neonate aortic smooth muscle cells when they were grown in medium containing 5% normal serum, 5% abeta serum, or 5% lipoprotein-deficient plasma ($\rho > 1.21$ g/ml). Smooth muscle cells, when grown in the presence of normal human serum, grew logarithmically for up to 7 days before entering a quiescent stationary phase of growth. However, cells grown in medium containing abeta serum or lipoprotein-deficient plasma grew logarithmically for only 2–3 days before entering a stationary, subconfluent phase. Similar growth responses to abeta serum, lipoprotein-deficient plasma, and normal human serum were observed for cultured neonatal and adult dermal fibroblasts (not shown). Although abeta serum was not as effective as normal serum in stimulating the proliferation and growth of either smooth muscle cells or dermal fibroblasts *in vitro*, this serum, as well as lipoprotein-deficient plasma, did maintain the cells in a viable state with prolonged culture.

Because abeta serum is devoid of the apoprotein-B-containing lipoproteins of density less than 1.063 g/ml (chylomicrons, VLDL, and LDL), the ineffectiveness of this serum to stimulate cell proliferation was compared to that of a normal serum fraction in which chylomicrons, VLDL, and LDL had been removed by ultracentrifugation ($\rho > 1.063$ g/ml fraction containing high density lipoprotein and $\rho > 1.21$ g/ml bottom fraction). As shown in Fig. 2, the $\rho > 1.063$ serum fraction stimulated cell proliferation and growth as effectively as whole serum, whereas its lipoprotein equivalent, abeta serum, showed only slight growth-promoting activity. Abeta plasma showed a similar absence of growth-stimulating activity (not shown). Addition of LDL to the $\rho > 1.063$ serum fraction resulted in no significant increase in the growth-promoting activity of this serum fraction.

Stimulation of Cell Growth by Serum Lipoprotein Supplementation. Because LDL has been shown to stimulate cell proliferation of cultured cells when added to medium containing lipoprotein-deficient plasma (7) or growth-limiting concentrations of serum (8), studies were carried out to determine whether serum lipoprotein supplementation of abeta serum would restore its growth-promoting capacity to that of normal serum. To test this, LDL prepared from normolipemic serum was added to abeta serum to give a cholesterol concentration equivalent to, or twice that of, 5% normal serum. LDL supplementation of abeta serum stimulated cell growth over that of abeta serum alone, but the cell density at 10 days was about half that observed for normal serum containing an equivalent amount of cholesterol (Fig. 3). Even when LDL-cholesterol was increased to twice that of normal serum (200 μg/ml of medium), cell proliferation still remained about half that observed for whole serum. Thus, although the addition of normolipidemic LDL to abeta serum resulted in the stimulation of cell proliferation and growth of both cultured human smooth muscle cells and dermal fibroblasts, the mitogenic effect of abeta serum could not be completely restored to that of normal serum. To further determine whether the discrepancy in the growth-promoting capacity between abeta serum and whole serum was due to differences in their cholesterol content, we

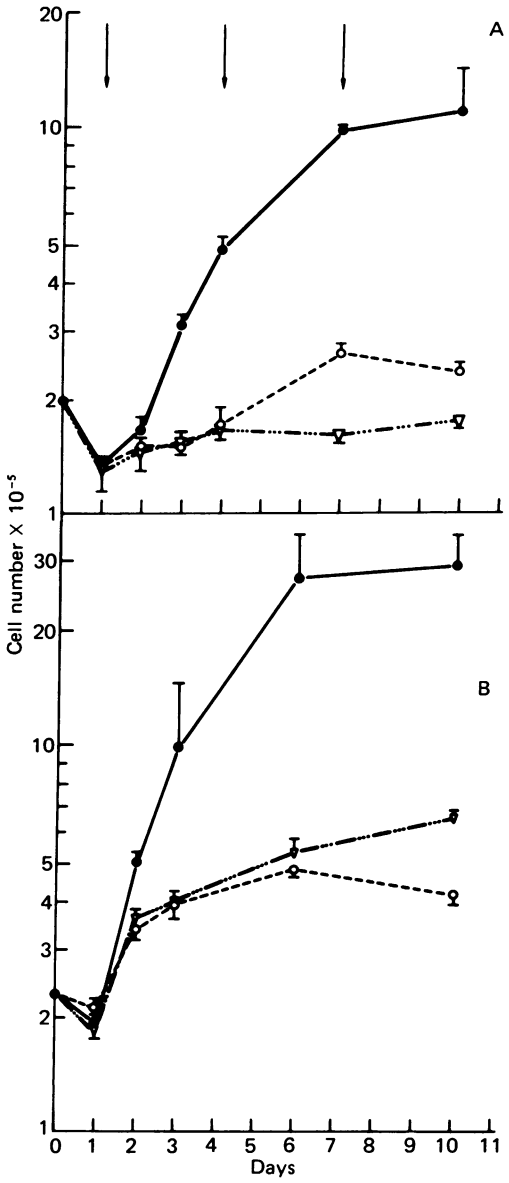

FIG. 1. Effect of normal human serum, abeta serum, and lipoprotein-deficient plasma on the proliferation and growth of adult (*A*) and neonate (*B*) human aortic smooth muscle cells. An equal number of cells were inoculated in 25-cm² tissue-culture flasks and grown in McCoy's medium containing 5% pooled human serum (●—●), 5% abeta serum (○---○), or 5% lipoprotein-deficient plasma ($\rho > 1.21$ g/ml) (▽—···—▽). Medium was changed on days 1, 4, and 7 (arrows) to maintain high nutrient levels. The cholesterol content of medium containing normal serum, abeta serum, or lipoprotein-deficient plasma was 97, 13, and 2 μg/ml, respectively. Each point represents an average cell count derived from duplicate flasks. Mean ± SEM are shown for each point.

grew cells in decreasing concentrations of normal serum. When cells were grown in 1% normal serum in which the total cholesterol concentration was 19 μg of cholesterol per ml of medium, 1% normal serum was 3 times more effective in promoting cell proliferation and growth than 10% abeta serum containing 26 μg of cholesterol per ml of growth medium (Fig. 4).

Restoration of Growth by Platelet Extract Supplementation. Initially, it was felt that the failure of abeta serum to stimulate cell proliferation and growth could not be attributed to a deficiency or abnormality of platelets because platelet count and platelet function, as measured by aggregometry and

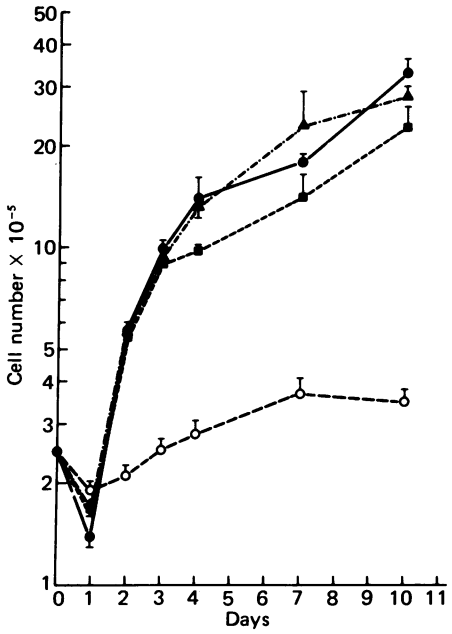

FIG. 2. Growth response of neonate dermal fibroblasts in culture to abeta serum compared to a serum fraction of $\rho > 1.063$ g/ml, in which chylomicrons, VLDL, and LDL had been removed by ultracentrifugation. The concentration of cholesterol in the medium was 97, 96, 22, and 13 μg/ml for 5% human serum (●), 5% serum with $\rho > 1.063$ plus LDL (▲), 5% serum with $\rho > 1.063$ (■), and 5% abeta serum (○), respectively. Growth conditions were similar to those described in Fig. 1.

[^{14}C]serotonin release in response to ADP, epinephrine, and collagen, was normal (16). However, because both abeta serum and normal serum were prepared identically in the presence of platelets and because abeta serum produced a growth response comparable to that of lipoprotein-deficient plasma, experiments were carried out to determine whether abeta serum exhibited an abnormality or deficiency in platelet growth factor(s). Five percent abeta serum was supplemented with a lysate of normal platelets, equivalent to the number of platelets in 5% normal serum (3.9×10^5 platelets per ml of serum) and then tested for its growth-promoting activity. Abeta serum supplemented with normal platelets stimulated the growth and proliferation of human smooth muscle cells as effectively as normal serum (Fig. 5). In contrast, normal lipoprotein-deficient plasma supplemented with a lysate of abeta platelets failed to stimulate the proliferation and growth of cultured smooth muscle cells (Fig. 6). However, the addition of a lysate of normal platelets to an identical sample of lipoprotein-deficient plasma resulted in the proliferation and growth of cells to a level achieved with normal serum.

DISCUSSION

This study clearly demonstrates that abeta serum did not stimulate the proliferation and growth of cultured human smooth muscle cells or dermal fibroblasts as effectively as normal serum. The growth-stimulating activity of this serum was comparable to the level achieved when cells were grown in the presence of lipoprotein-deficient plasma of $\rho > 1.21$ g/ml (Fig. 1). The inability of abeta serum to promote cell proliferation could not be wholly attributed to the absence of the apoprotein-B-containing lipoproteins because the addition of LDL up to twice the cholesterol concentration of normal serum did not restore its growth-promoting activity to that of normal serum (Fig. 3). Moreover, the addition of cholesterol in the form of intermediate density lipoproteins ($\rho > 1.006$–1.019 g/ml)

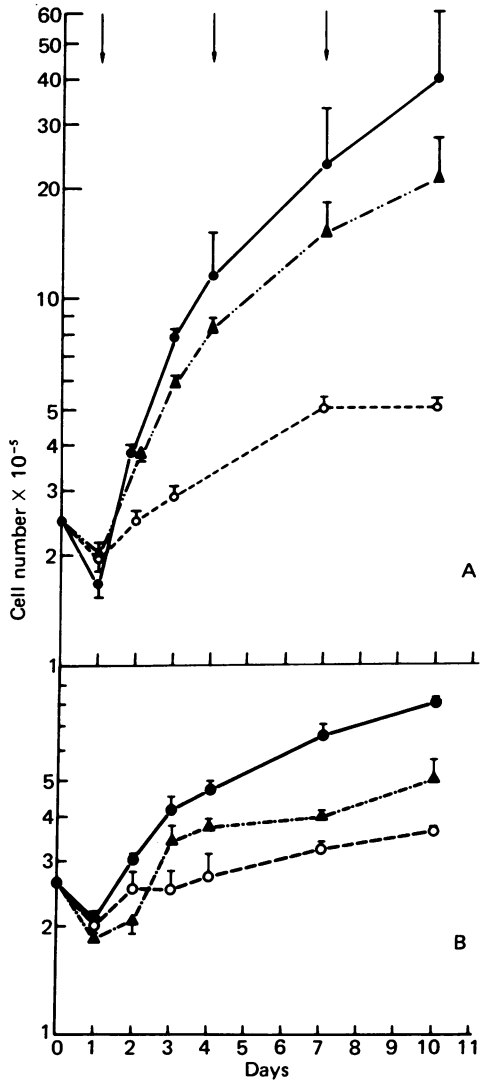

FIG. 3. Response of dermal fibroblasts (*A*) and smooth muscle cells (*B*) to normolipemic LDL supplementation of abeta serum. Dermal fibroblasts were grown in medium containing abeta serum supplemented with LDL to give a final cholesterol concentration twice that of normal serum (195 compared to 97 μg/ml). Smooth muscle cells were cultured in LDL-supplemented abeta serum at a cholesterol concentration equivalent to normal serum (97 μg/ml). See legend to Fig. 1 for experimental details. ●—●, 5% human serum; ▲—·—▲, 5% abeta serum plus LDL; ○---○, 5% abeta serum.

to abeta serum also resulted in a submaximal growth response similar to that obtained with LDL supplementation (not shown).

An additional study showed that the inability of abeta serum to support growth could not be attributed to a general reduction in total serum cholesterol because 1% normal serum containing one-third less cholesterol promoted cell proliferation and growth 3 times more effectively than 10% abeta serum (Fig. 4).

Several studies have shown that the full mitogenic effect of serum cannot be completely recovered by recombining lipoprotein-deficient plasma ($\rho > 1.21$) with individual lipoproteins (8, 10). Approximately half of the growth-promoting effect of lipoprotein-deficient plasma was restored with VLDL or LDL supplementation. In these studies, lipoprotein supplementation of abeta serum produced a cell growth response comparable to the level observed for lipoprotein supplementation of lipoprotein-deficient plasma.

Because abeta serum is devoid of the apoprotein-B-con-

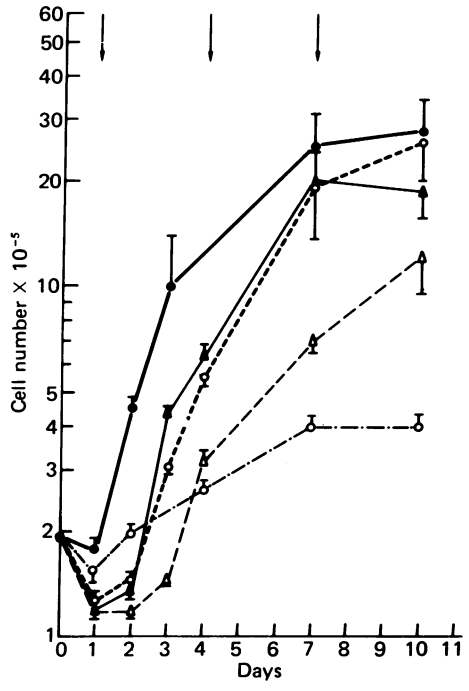

FIG. 4. Growth response of dermal fibroblasts to different concentrations of normal serum in the culture medium. Cells were pipetted into 25-cm² flasks and grown in McCoy's 5a medium supplemented with (cholesterol concentrations per ml of medium): ●—●, 10% human serum (194 μg/ml); O---O, 3% human serum (58 μg/ml); ▲—▲, 5% human serum (97 μg/ml); △---△, 1% human serum (19 μg/ml). In one experimental series, cells were grown in 10% abeta serum (O-·-O). Arrows denote medium change.

taining lipoproteins, the growth-promoting activity of this serum was compared to a normal serum fraction in which the chylomicrons, VLDL, and LDL had been removed by ultracentrifugation. In contrast to abeta serum, this normal serum fraction promoted cell proliferation and growth as effectively as whole serum (Fig. 2). No further stimulation of growth was observed when LDL was added back to this fraction, indicating that all of the growth-promoting activity of whole human serum was contained in the serum fraction of $\rho > 1.063$ composed of high density lipoprotein and the $\rho > 1.21$ bottom fraction.

The difference in the growth-stimulating activity between abeta serum and the normal serum fraction of $\rho > 1.063$ indicated that abeta serum, in addition to being devoid of the apoprotein-B-containing lipoproteins, may be also deficient in some platelet growth factor(s). That this was the case was shown by adding a lysate of normal platelets to abeta serum. This supplementation restored the growth-promoting activity of abeta serum to that of normal serum (Fig. 5). Furthermore, the growth-promoting activity of normal lipoprotein-deficient plasma was not restored to normal after the addition of a lysate of abeta platelets, whereas a similar supplementation with normal platelets promoted cell proliferation and growth as effectively as normal serum (Fig. 6). These studies indicate that the platelets from the blood of patients with abetalipoproteinemia are deficient in some platelet releasable growth factor(s). This apparent deficiency in a releasable platelet growth factor does not appear to be due to a reduced platelet count or platelet function, because platelet count and platelet function, as measured by aggregometry and [^{14}C]serotonin release in response to ADP, epinephrine, and collagen, were normal (16). Prostaglandin synthesis in these platelets also appeared to be normal, as indicated by malondialdehyde production after

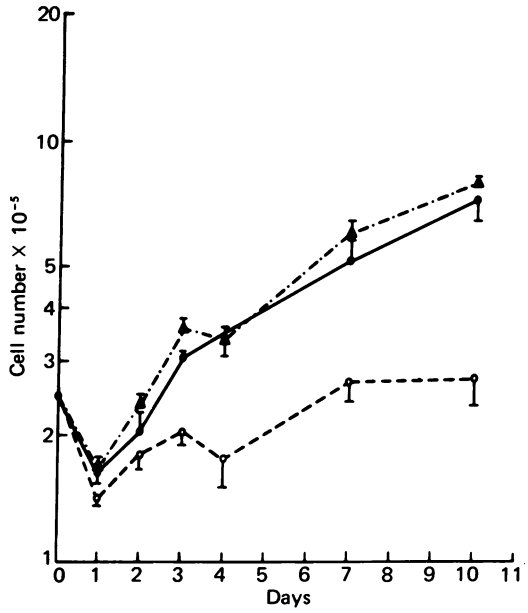

FIG. 5. Growth response of aortic smooth muscle cells to abeta serum supplemented with normal human platelets. A suspension of normal platelets in saline (1.1×10^6 platelets per ml) was lysed by freezing and thawing, and 1.8 ml of this lysate was added to 5 ml of abeta serum to simulate the number of platelets lysed during the preparation of normal serum (3.9×10^5 platelets per ml). This supplemented abeta serum was used to make up medium containing 5% serum. ▲-·-▲, 5% abeta serum plus normal platelets; ●—●, 5% normal serum; O---O, 5% abeta serum.

stimulation with *N*-ethylmaleimide. Despite low plasma cholesterol levels in abetalipoproteinemic subjects, the content of this lipid was normal in their platelets (16). Although the con-

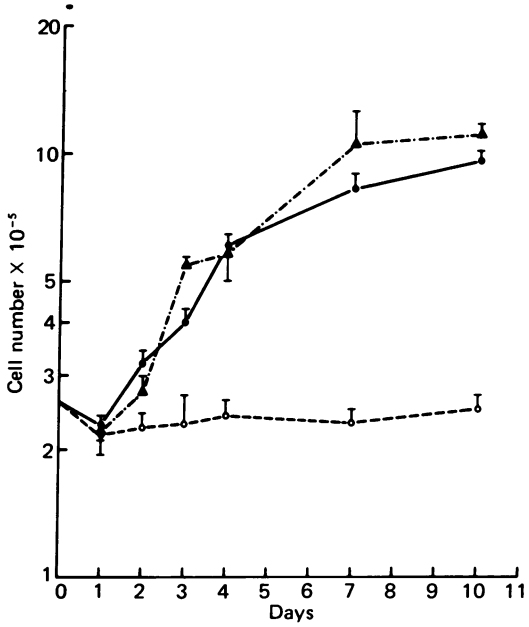

FIG. 6. Growth response of cultured dermal fibroblasts to lipoprotein-deficient plasma supplemented with a platelet lysate from abetalipoproteinemic blood. A suspension of platelets from normal or abetaliproproteinemic blood was lysed by freezing and thawing, and a volume of lysate from each, which was equivalent to the number of platelets lysed during the preparation of normal serum or abeta serum, was added to lipoprotein-deficient plasma. Cells were counted on the days indicated. See legend to Fig. 5 for details. ▲-·-▲, 5% plasma ($\rho = 1.21$) plus normal platelets; ●—●, 5% human serum; O---O, 5% plasma ($\rho = 1.21$) plus abeta platelets.

centration of vitamin E was found to be reduced in both plasma and platelets of patients with abetalipoproteinemia (16), vitamin E supplementation of abeta serum had no effect in stimulating its proliferative capacity (not shown). Furthermore, Goodnight *et al.* (16) concluded that these reduced levels of vitamin E did not interfere with normal platelet function.

Recent observations by Witte *et al.* (10) indicate that release of platelet growth factor and of serotonin may occur by different mechanisms. Their evidence suggests that the platelet growth factor is stored in the light α granules and is released by low doses of thrombin. In contrast, serotonin, which is localized in the dense granules, is released by higher doses of thrombin. Thus, although platelets isolated from the blood of patients with abetalipoproteinemia may contain normal dense granules and exhibit normal serotonin release, they may contain deficient or abnormal α granules and, therefore, have reduced or abnormal platelet growth factor.

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