PREPARATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST HUMAN APOLIPOPROTEIN E

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Apolipoprotein E (apoE) is one of the most important apo-proteins of the blood lipid transport system. As a ligand of apoE- and apoB,E-receptors of the liver cells, apoE is essential for the uptake of remnants both of chylomicrons and of very low-density lipoproteins (VLDL), and also of apoE containing low-density lipoproteins (LDL) by the liver. Disturbance of interaction of apoE with the receptor, in the presence of unfavorable factors, leads to type III hyperlipoproteinemia. ApoE also is involved in the reverse transport of cholesterol, regeneration of nerve tissue, and also, possibly, immunoregulation [9]. A characteristic feature of apoE is the presence of several isoforms of this apo-protein with different parameters of interaction with receptors [10].

The aim of the present investigation was to obtain antibodies (AB) to apoE in order to study the structure of apoE and its interaction with lipid and receptor, and also to determine isoforms and the content of apoE in human blood plasma for prognostic purposes.

EXPERIMENTAL METHOD

ApoE was purified by the method in [1] from VLDL of human blood plasma. Monoclonal antibodies (McAb) to apoE as antigen (Ag) were obtained as in [7], in the modification in [3]. The specificity of Ag was tested by immunodiffusion and immunoblotting after isoelectric focusing of human serum proteins. BALB/c mice were immunized three times by intraperitoneal injection of 100 μg apoE, with the addition of one-third of its volume of Freund's complete adjuvant, during the first two immunizations. For hybridization, the mouse spleen and myeloma cells of the X63-Ag 8.6.5.3 line, in the logarithmic phase of growth with a density of $(5\text{-}6)\cdot10^5$ cells/ml were used. The ratio of splenocytes obtained from an immune mouse and myeloma cells was 5:1. Fusion was carried out with the aid of polyethylene-glycol with mol. wt. 3350. After hybridization the cells were suspended in medium RPMI-1640, washed twice, then seeded in HAT medium in 96-well planchets, with $2\cdot10^5$ cells per well. The medium was changed for one containing GT 5 days after the appearance of the first hybrid clones. On the 12th-14th day the hybrids were analyzed for ability to secrete Ag. Screening was carried out by ELISA. The positively responding primary populations were cloned. As they grew, the clones were transferred to 24-well planchets, in flasks with an area of 25 cm^2, and later into flasks with an area of 75 cm^2. Hybrid cells ($5\cdot10^6$) were injected intraperitoneally into mice, treated beforehand with 0.5 ml of the immunodepressant pristan (tetramethylpentadecane). Ascites fluid formed 10-20 days after injection of the cells was used as the source of the McAb. The Ab were purified by salting the immunoglobulins out of the ascites fluid, and then purifying them further on protein-A-sepharose [11]. The dissociation constant of the Ag-Ab complex was determined in solution, using a technique of enzyme immunoassay by a modified method [4]. McAb in a constant concentration of 0.4-1.0 nM were titrated with different concentrations of apoE. The reaction was carried out in 0.1 M phosphate buffer (2 mM EDTA, pH 7.8), with the addition of 0.5% human serum

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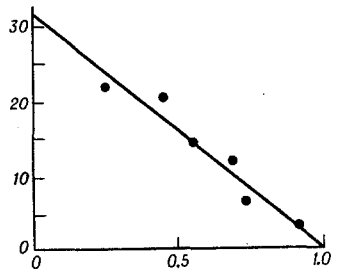

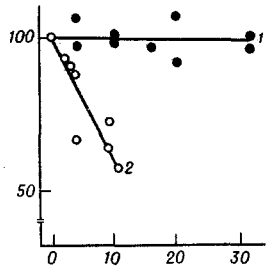

Fig. 1 Fig. 2

Fig. 1. Binding of apoE with McAb 3D12F11 in modified Scatchard coordinates. Abscissa, fraction of McAb bound with apoE (Ab_B); ordinate, ratio of fraction of bound McAb to concentration of free apoE (in $M^{-1} \cdot 10^7$). Concentration of free apoE calculated by equation $[apoe]_F = [apoE]_{tot} - Ab_B \cdot [McAb]_{tot}$. Dissociation constant K_d) reciprocal of intercept along axis of ordinates.

Fig. 2. Effect of Ab against apoE on binding of apoE with heparin-sepharose. Ordinate, fraction of apoE (in %) bound with heparin-sepharose in presence of IgG McAb (1) or Fab-fragments of polyclonal antibodies (2); abscissa, ratio of concentration of anti-apoE (expressed in concentration of Fab fragments) to apoE concentration (moles/moles).

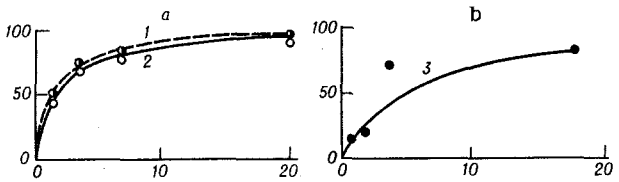

Fig. 3. Outflow of carboxyfluorescein from liposomes. Ordinate, outflow of contents of liposomes (in %); a) induced apoE in absence of McAb (2) or in presence of a tenfold excess of McAb (1); abscissa, apoE concentration (in nM); b) McAb-induced outflow (3); abscissa, McAb concentration (in nM).

albumin (HSA) overnight at 20°C. To each well of the planchet for enzyme microimmunoassay ("Dynatech," USA) was added 0.1 μg of apoE in 10 mM carbonate buffer, pH 9.6, and the contents were incubated overnight at 20°C. Next followed washing with phosphate buffer with 0.5% Tween-20, and the residual binding sites were blocked with 0.5% HSA in phosphate buffer for 1 h at 20°C. The planchet was then washed and the specimen to be measured was introduced and incubated for 15 min at 20°C. The bound McAb were revealed by peroxidase labeled secondary Ab against mouse IgG (1:500, "Amersham"), and the substrate for the peroxidase was O-phenylenediamine. The reaction was stopped by addition of 2.5 M H_2SO_4. A preliminary calibration curve showed that ingestion is a linear function of Ab concentration within the region of Ab concentrations measured. Some Ab, bound from solution with Ag immobilized on the planchet, accounted for about 4% of the added amount and did not affect the shift of equilibrium in the solution. The results are shown within modified Scatchard plot coordinates: dependence of $Ab_B[apoE]_F$ on Ab_B, where Ab_B indicates the fraction of Ab bound in solution in the form of an Ag—Ab complex, and $[apoE]_F$ denotes the concentration of free apoE. The value of the dissociation constant K_d was calculated as the reciprocal of the coordinate of the point where the Scatchard plot intersects the axis of ordinates. Binding of apoE with heparin-sepharose was determined by a modified method [13]. The heparin-sepharose and control sepharose were obtained by the method in [2] from CNBr-activated sepharose 4B. ApoE, labeled with fluores-

cein isothiocyanate (FITC) in the ratio of 1 mole FITC to 1 mole apoE (apoE-F) was obtained by the method in [12]. To prepare the samples 2 μg of apoE-F was mixed with McAb against apoE and the volume of the sample made up to 0.3 ml. The reaction was carried out in 50 mM NaCl in 5 mM Tris-HCl, pH 7.4, with 2 mg/ml of HSA for 1.5 h at 20°C. The samples were incubated for 1 h at 20°C. After incubation the sepharose was sedimented by centrifugation and 100-μl samples of the supernatant were taken. The volume of the sample was made up to 0.6 ml and fluorescence was measured in 5% SDS. The action of apoE on the outflow of carboxyfluorescein from liposomes prepared from dipalmitoylphosphatidylcholine (DPPC) was determined by the method in [6]. For the experiments to study the outflow of carboxyfluorescein from liposomes, the dye had to be purified. This was done initially by hydrophobic chromatography on Sephadex LH-20-100 (Sigma, USA) [6], followed by high-efficiency liquid chromatography on a "Lichrosorb RP-8" column (LKB, Sweden), in 5% acetonitrile. The liposomes were prepared by sonication of 1 mg DPPC in 1 ml of 0.05 M carboxyfluorescein/10 mM HEPES, pH 7.4/0.05 M NaCl. The liposomes were separated from the free dye on sepharose CL-6B. The apoE, preincubated for 2 h at 37°C in the absence or presence of a tenfold excess of McAb, was added to the liposomes (0.03 μg DPPC in 1 ml) in ice, after which the sample was introduced into a fluorometric cuvette, thermostated at 43°C. When the phase transition temperature was reached, carboxyfluorescein was released from the liposomes and the previously quenched fluorescence flared up. The fraction of carboxyf luorescein released was calculated relative to the total content of carboxyfluorescein, determined after destruction of the liposomes with Triton X-100. Protein was determined by the method in [8] in the presence of 5% SDS.

EXPERIMENTAL RESULTS

Of the various clones obtained, for further characterization we chose clone 3D12F11, because of the high affinity of the McAb produced by it. Binding with protein-A-sepharose showed that McAb 3D12F11 belong to the IgG subclass, for they were eluted from the column at pH 6.0 [11]. The specificity of the Ab was tested by isoelectric focusing of the plasma proteins followed by immunoblotting [5]. The Ab obtained bound only with proteins corresponding in their position to isoforms of apoE. These results show that the Ab obtained can be used for phenotyping samples of plasma relative to apoE. The dissociation constant K_d of the Ag—Ab complex was determined by enzyme immunoassay, and was 3.5 ± 0.5 nM (n = 5, Fig. 1). The data in Fig. 2 show that McAb, even with a 30-fold excess, did not affect binding of apoE with heparin-sepharose, whereas polyclonal Ab to apoE significantly reduced this finding. This indicates that the antigenic determinant for McAb 3D12F11 does not include a heparin-binding site. The importance of this conclusion will be obvious, for the heparin-binding site is located in the receptor-binding domain of apoE [9]. McAb had no significant effect on insertion of apoE into liposomes (Fig. 3), for they did not reduce induction of dye release. McAb likewise led to release of the contents of the liposomes, although to a much lesser degree than apoE. Thus the antigenic determinant for McAb 3D12F11 is evidently located in the exposed part of the apoE molecule. Consequently, McAb 3D12F11 can be used to investigate binding of apoE with the receptor and for quantitative determination of apoE in human blood plasma.

LITERATURE CITED

1. A. D. Dergunov, L. P. Aniskovich, and V. V. Shuvaev, Byull. Éksp. Biol. Med., **110**, No. 7, 42 (1990).
2. Affinity Chromatography: Principles and Methods, Pharmacia, Uppsala (1978).
3. R. L. Davidson and P. S. Gerals, Somat. Cell Genet., **2**, 165 (1976).
4. B. Friguet, A. F. Chaffotte, L. Djavadi-Ohaniance, and M. E. Goldberg, J. Immunol. Meth., **77**, 305 (1985).
5. R. E. Greeg, G. Ghiselli, and H. B. Brewer, jr., Biochim. Biophys. Acta, **794**, 333 (1984).
6. R. D. Klausner, R. Blumenthal, T. Innerarity, and J. Weinstein, J. Biol. Chem., **260**, 13,719 (1985).
7. G. Kohler and C. Milstein, Nature, **256**, 495 (1975).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem., **193**, 265 (1951).
9. R. W. Mahley, T. L. Inerarity, S. C. Rall, jr., and K. H. Weisgraber, J. Lipid Res., **25**, 1277 (1984).
10. R. W. Mahley, Science, **240**, 622 (1988).
11. Separation News, Pharmacia, **13**, No. 5, 1 (1986).
12. M. Steiner, Biochim. Biophys. Acta, **805**, 53 (1984).
13. K. H. Weisgraber, S. C. Rall, R. W. Mahley, et al., J. Biol. Chem., **261**, 2068 (1986).