Vol. 87, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1979 Pages 121-127

RADIOIMMUNOLOGICAL STUDY OF THE SURFACE PROTEIN OF THE HUMAN SERUM LOW-DENSITY LIPOPROTEIN: COMPARISON OF THE NATIVE PARTICLE AND THE PRODUCTS OBTAINED BY TRYPTIC TREATMENT*

Sonia Goldstein and M.John Chapman
With the technical assistance of D.Lagrange and A.M.Bridoux

Unité 35 (Unité de Recherche sur le Métabolisme des Lipides),
Institut National de la Santé et de la Recherche Médicale,
Hôpital Henri Mondor, 94010 Créteil, France

Received January 31,1979

SUMMARY

The present report describes radioimmunological studies of the native low-density lipoprotein from human serum, and of the products obtained by limited tryptic treatment,i.e.a protein-depleted particle lacking some 20% of the original protein moiety and a peptide fraction of low molecular weight (<5000). The liberated peptides were highly immunogenic and elicited antibodies which reacted with both the native and protein-deficient lipoprotein particles. Moreover these peptides exhibited competitive reactivity with [^{125}I]-labelled low-density lipoprotein in binding with homologous antisera, and with antisera to the native and trypsin-treated lipoproteins. These findings suggest that the peptides liberated from low-density lipoprotein by tryptic digestion contain the major antigenic site(s) of the molecule. Consideration of the nature of the competitive displacement of radiolabelled low-density lipoprotein from antisera to low-density lipoprotein, to the trypsinised lipoprotein and to the peptide fraction indicate that a marked repetition of the antigenic site(s) occurs in the structure of the protein moiety, a possibility consistent with the recurrence of similar subunits in the apoprotein of low-density lipoprotein.

INTRODUCTION

Although there is an abundance of data on the molecular organisation of the human serum low-density lipoprotein (1,2), the structure of its major protein component, apolipoprotein B, remains controversial. Thus, estimates of its molecular weight range from 8,000 to 270,000 (1,2) and recent studies (3,4) suggest that low-density lipoprotein apoprotein may consist of segments of very similar amino acid composition.

*Partially supported by C.R.L. No.77.5.021.5 from Institut National de la Santé et de la Recherche Médicale and by Grant No.78.7.2598 from the Délégation Générale à la Recherche Scientifique et Technique.

Abbreviations: LDL, low-density lipoprotein; T-LDL, trypsinised low-density lipoprotein; T-peptides, peptides liberated from LDL by tryptic treatment; ID, immunodiffusion; IEP, immunoelectrophoresis; PBS, phosphate-buffered saline.

0006-291X/79/050121-07$01.00/0

Copyright © 1979 by Academic Press, Inc.
All rights of reproduction in any form reserved.

A previous report from this laboratory (5) has shown that limited tryptic digestion of low-density lipoprotein (LDL) may, under certain conditions, yield a protein depleted particle (T-LDL), whose apoprotein contains subunits ranging in mol.wt. from 160,000 - 10,000. In addition, the protein liberated by trypsin (T-peptides), consists of a number of small peptides (mol.wt. < 5000), which are apparently derived from the surface of LDL (6).

The aim of the present study was to establish whether the trypsin-releasable peptides and the protein-deficient LDL particle share common antigenic sites, and to determine their relationship to the protein of the native particle.

MATERIALS AND METHODS

Human serum LDL was isolated in the density interval 1.024-1.045 g/ml by sequential flotational ultracentrifugation (7), under conditions previously described (8). The LDL preparations were submitted to a limited tryptic treatment; the tryptic digest was placed on a Sephadex G-75 column, yielding two fractions, a trypsin-treated LDL (T-LDL) depleted of about 20% of its original protein, and a peptide fraction (T-peptides) of mol.wt. < 5000 (5).

Several antisera to native LDL, two antisera to different T-LDL and two antisera to different T-peptide preparations were produced in rabbits by intradermal injections, at several sites, of 300 µg LDL protein and 500 µg T-LDL and T-peptide protein. A single booster injection was given after 3 weeks for LDL and T-LDL, with 3 booster injections at intervals of 3 weeks for the T-peptides. After estimation of the antibody titre for each antigen as previously described (8), two different antisera were employed for each antigen.

Double immunodiffusion (ID) and immunoelectrophoresis (IEP) were carried out by the techniques of Ouchterlony (9) and Scheidegger (10) respectively, under conditions previously described (8).

For the radioimmunological study, LDL (2mg) in 0.5 ml of 0.1 M glycine buffer (pH 10), was iodinated according to a modification (11) of McFarlane's iodine monochloride technique (12). After a tenfold dilution of the labelled LDL with the glycine buffer, free iodide was removed by dialysis against 10 liters of a solution containing 0.15 M NaCl, 10 mM Tris and 0.01% EDTA, at pH 7.4 for 20 hours at 4°C, with 8-10 changes of the dialysate. The specific activity of the [^{125}I] -labelled LDL ranged between 130 and 250 cpm/ng protein, and 9-12% of the radioactivity of this LDL remained in the supernatant after addition of trichloroacetic acid. The extent of lipid labelling did not exceed 8%. After 1/10 dilution in the glycine buffer, the labelled LDL was stored in 0.5 ml aliquots at 4°C. Such radio-labelled LDL was found to be stable for 4-6 weeks. Some 95-98% of the radioiodinated LDL was found when incubated with antibody excess, thus establishing the integrity of the molecule.

The radioimmunoassay (RIA) was performed by the double antibody method as described by Felber (13). All the dilutions were performed in phosphate-buffered saline (PBS), containing 0.01% sodium azide, 0.01% EDTA and 1% bovine serum albumin, at pH 7.5. For the determination of the dilution of the antiserum to be used, 0.1 ml of antiserum in serial dilutions of from 1/10 to 1/50,000 were reacted with 0.1 ml radiolabelled LDL (in a dilution corresponding to approx. 50-75 ng protein and 10,000-12,000 cpm. Standards and samples, made up to a total volume of 0.5 ml, were incubated for 48 hr. at 4°C. The second antibody reaction consisted of the addition of 0.1 ml of

a sheep antiserum to rabbit gamma-globulin, diluted 1/10 (Institut Pasteur, Paris). After 24 hr. at 4°C and subsequent washings with 1 ml of ice-cold PBS, the tubes were centrifuged at 2000 g at 4°C for 20 min.; the supernatant was subsequently discarded and the precipitates counted for 1-2 min. in an Autogamma spectrometer (model CG 4000, Intertechnique). The experiments were done in duplicate or triplicate. The results were plotted to give an antigen binding curve, and the dilution of antiserum corresponding to a 45-50% binding of radiolabelled LDL was retained for the competition assay; these dilutions were 1/5000 and 1/4000 respectively for the two antisera to LDL, 1/4000 for the antisera to T-LDL, and 1/500 and 1/400 for the two antisera to the T-peptide fraction.

For the inhibition of labelled antigen binding, aliquots (0.1 ml) of antiserum at the chosen dilution were reacted with progressive amounts of the competitive antigen in a volume of 0.1 ml, i.e. 5 - 350 ng of LDL or T-LDL protein, and 300 - 5000 ng of T-peptide protein. After incubation for 5 to 7 days at 4°C, 0.1 ml of labelled LDL (10,000 - 12,000 cpm) was added; after a further 24 hrs. of incubation at 4°C, the second antibody was added and the experiment completed as described above. Controls for non-specific binding of labelled LDL were included; they typically represented about 3-5% of the total radioactivity detectable in each assay (6% for the highest amounts of T-peptides), and were subtracted from the corresponding experimental values. The results of the competition assay were plotted as B/B_0 (B = cpm in precipitate in presence of unlabelled antigen; B_0 = cpm in precipitate in the absence of the unlabelled antigen) against the protein concentration of the unlabelled antigen, on a semilog. scale.

RESULTS AND DISCUSSION

In confirmation of a previous report in which the protein-deficient LDL particle resulting from tryptic treatment was characterised (5), the amino acid compositions of the native LDL's and of the corresponding trypsin-treated fractions (T-LDL) were essentially indistinguishable (data not shown). The liberated peptides were however enriched in lysine, arginine and valine, and displayed less aspartic acid, glutamic acid and leucine than native and T-LDL; the peptide fraction contained some 30% or more of the sialic acid content of LDL (unpublished observations), and trace amounts of free fatty acids. No alteration of the phospholipid components of LDL was detected in the T-LDL fraction.

All the rabbits injected with LDL, T-LDL and T-peptides produced antibodies; although the titres of the antisera against T-peptides were the weakest, both LDL and T-LDL reacted upon immunodiffusion and immunoelectrophoresis with all the antisera employed (Fig.1, ID pattern). The T-peptide fraction failed however to react with any of these antisera, even when large amounts of antigen or antiserum were employed (up to 200 µg of T-peptide instead of 10-20 µg LDL or T-LDL protein, and 200 µl instead of 100 µl of antiserum.

Radioimmunoassay was performed with five different preparations of LDL, T-LDL and T-peptides. The two sets of antisera directed to each antigen

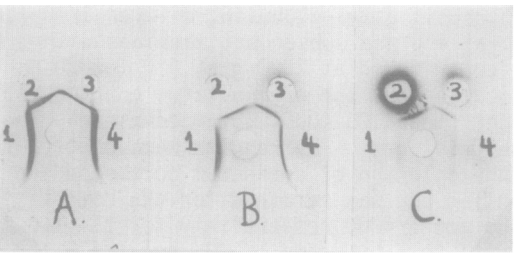

Fig.1. Immunodiffusion pattern of LDL and T-LDL reacting with antisera to LDL, to T-LDL and to T-peptides. Central wells: A.Antiserum to LDL; B. Antiserum to T-LDL; C.Antiserum to T-peptides. Peripheral wells: 1 and 3, LDL (20 µg protein); 2 and 4, T-LDL (20 µg protein). Sudan black staining.

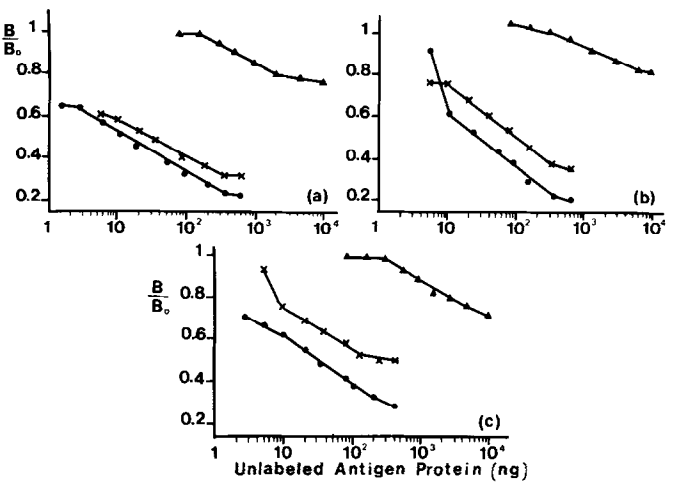

Fig.2. Competitive displacement of [^{125}I] -labelled LDL by unlabelled LDL (●); T-LDL (✕); and T-peptides (▲). B/B_0 (ordinate) was calculated as described in "Materials and Methods" and plotted against increasing concentrations of unlabelled protein (abcissa). (a) Antiserum to LDL. (b) Antiserum to T-LDL. (c) Antiserum to T-peptides.

gave essentially identical results. Representative competitive displacements of [^{125}I] -labelled LDL from anti-LDL, anti-T-LDL and anti-T-peptide antisera by unlabelled LDL, T-LDL and T-peptides are shown in Fig.2. Means of the experiments with the five different preparations are reported in Table 1. Several points warrant comment: (i) All three fractions, i.e. LDL, T-LDL and T-peptides, compete with labelled LDL in binding to the antisera, but markedly higher amounts of T-peptides are needed for the displacement of the labelled antigen (500-5000 ng of T-peptide protein in comparison to 10-300 ng of LDL or T-LDL protein (Fig.2); (ii) The binding capacity of the T-peptides, even at the highest protein concentrations does not reach 25%, whereas that of

TABLE I

COMPARISON OF THE % INHIBITION OF THE BINDING OF [^{125}I]-LABELLED LDL, T-LDL AND T-PEPTIDES AS DETERMINED BY RADIOIMMUNOASSAY.

Unlabelled antigen		% Inhibition of binding of [^{125}I] -labelled LDL		
		Antisera to:		
		LDL	T-LDL	T-peptides
LDL	10 ng	41 (± 5.6)	35 (± 9.6)	50 (± 14.0)
	300 ng	70 (± 2.6)	70 (± 3.7)	71 (± 10.7)
T-LDL	10 ng	33 (± 5.8)	40 (± 7.6)	30 (± 9.5)
	300 ng	60 (± 3.5)	61 (± 3.3)	53 (± 14.2)
T-peptides	500 ng	11 (± 7.6)	0	10 (± 7.9)
	5000 ng	17 (± 7.8)	11 (± 3.8)	22 (± 5.6)

For each antiserum, results are the means ± standard deviation of two or three separate experiments performed on five different LDL and the corresponding trypsin-treated fractions; individual assays were made in duplicate or triplicate.

both LDL and T-LDL attains some 70% (Fig.2 and Table 1). This is in accordance with data on the effect of fragments of an antigen on the binding of the entire labelled antigen to the antibody (14). Thus, in the case of ACTH, different polypeptide fragments elicited a response amounting to 70-0.2% of the activity of the whole molecule (14); (iii) LDL competes strongly with the labelled antigen in binding to the three antisera; T-LDL also competes in binding to antisera to LDL and T-LDL, and less in binding to the antiserum to the T-peptides. The displacement exerted by the T-peptide fraction is inferior to that of LDL and T-LDL in all cases, but is however greater when binding to its homologous antiserum. (Fig.2 and Table 1); (iv) The slopes of the lines plotted for the different antisera may provide valuable information on the relationship between several antigens. According to Hunter (14), the behaviour of immunologically-related proteins in antigen addition curves may be classified either as a reaction of identity (parallelism of the slope with small differences in the quantities required to give the same depression in binding the labelled antigen) or as complete or incomplete cross-reactions (lack of parallelism or marked differences in the quantities of unlabelled competitor, or changes in the three-demensional arrangement). Upon consideration of the slopes obtained with the three antigens, it is evident that in the reaction with the antiserum to LDL (Fig.2a), the curves given by addition of increasing amounts of T-LDL and T-peptides have nearly the same

slope as that of the native protein throughout the entire concentration range. In this case, the three antigens may be considered as presenting reactions of complete or partial identity in their immunological behaviour. In the reaction with the antiserum to T-LDL (Fig.2b), the lines corresponding to LDL and T-LDL also exhibit parallel slopes; the slope for the T-peptides is however shallower than that of the other antigens, thus indicating that the T-peptides are capable of competing with the labelled antigen for all the binding sites on the antibody, but that their affinity for these antibodies is diminished.

Our previous studies have shown that all the polypeptide chains of the protein moiety of human serum LDL (essentially apolipoprotein B), appear to be available to some extent to trypsin (5,6). We thus considered it of interest to establish the immunological relationship between the trypsin-releasable peptides (mol.wt. < 5000), the protein-depleted LDL particle (T-LDL) whose apoprotein exhibits several subunits of mol.wt.160,000-10,000, and the native particle, whose apoprotein displays a mol.wt. $> 250,000$ under the same conditions (5).

We now show that the liberated peptides are highly immunogenic and ellicit antibodies which react strongly both with the native LDL and with the protein-deficient particle. Moreover, these peptides exhibit a competitive reactivity with [^{125}I]-labelled LDL in binding with antisera to native LDL, to T-LDL and to T-peptides. Such findings suggest that the peptides detached from the LDL particle by limited tryptic treatment contain the major antigenic site(s) of the molecule. Their high immunogenicity, considered together with the strength of the immunological reactivity exhibited by both fractions (T-LDL and T-peptides) obtained by tryptic digestion with all the antisera studied, is suggestive of a marked repetition of this antigenic site(s) in the whole particle, and may imply the recurrence of identical polypeptide chains in the protein moiety of LDL. It is also of note that the T-peptide fragments are enriched in lysine, an amino acid which often participates in the immunologically-reactive regions of polypeptide antigens (15).

In conclusion, our present findings are consistent with the recent data of Chen and Aladjem (3) and of Deutsch et al.(4), which suggest the presence either of one or of a small number of fundamental polypeptide subunits of similar amino acid composition in the protein moiety of LDL.

REFERENCES

1. MORRISET J.D., JACKSON R.L. and GOTTO jr.A.M. (1975) Ann. Rev. Biochem. 44, 183-207.

2. SMITH L.C., POWNALL H.J. and GOTTO jr.A.M. (1978) Ann.Rev.Biochem.47, 751-777.
3. CHEN C.H. and ALADJEM F. (1978) Biochem.Med. 19, 178-187.
4. DEUTSCH D.G., HEINRIKSON R.L., FOREMAN J. and SCANU A.M. (1978) Biochem. Biophys. Acta 529, 342-350.
5. CHAPMAN M.J., GOLDSTEIN S. and MILLS G.L. (1978) Eur.J.Biochem. 87, 475-488.
6. LAGGNER P., CHAPMAN M.J. and GOLDSTEIN S. (1978) Biochem. Biophys.Res. Commun. 82, 1332-1339.
7. HAVEL R.J., EDER H.A. and BRAGDON J.H. (1955) J. Clin.Invest.34, 1345-1353.
8. CHAPMAN M.J. and GOLDSTEIN S. (1976) Atherosclerosis 25, 267-291.
9. OUCHTERLONY O. (1964) In: Immunological Methods, J.F. ACKROYD ed., pp. 54-91, Blackwell, London.
10. SCHEIDEGGER J.J. (1955) Int.Arch.Allergy Appl.Immunol. 7, 103-110.
11. BILHEIMER D.W., EISENBERG S. and LEVY R.I. (1968) Proc.Natl.Acad.Sci. USA. 61, 636-643.
12. McFARLANE A.S. (1958) Nature 182, 53.
13. FELBER J.P. (1974) In: Methods of Biochemical Analysis, vol.22, D.GLICK ed., pp. 1-94, Interscience, New York.
14. HUNTER W.M. (1978) In: Handbook of Experimental Immunology, vol.1, D.M.WEIR ed., pp.14.1-14.40, Blackwell, London.
15. KABAT E.A. (1978) Structural Concepts in Immunology and Immunochemistry pp.9-26., Holt, Rinehart and Winston, INC., New York.