Inhibition of Lymphoproliferation by Hyperlipoproteinemic Plasma

CAROLINE C. WADDELL, O. DAVID TAUNTON, and JEREMIAH J. TWOMEY

From the Hematology and Atherosclerosis and Lipoprotein Research Sections and the Immunohematology Research Laboratory, Department of Medicine, Baylor College of Medicine, Veterans Administration and The Methodist Hospitals, Houston, Texas 77031

ABSTRACT Plasma from patients with primary type IV or V hyperlipoproteinemia inhibited [^{3}H]thymidine incorporation by cultured mononuclear leukocytes. This previously unreported abnormality affected mononuclear leukocytes from patients with type IV or V hyperlipoproteinemia and from normal subjects. Patient cells incorporated [^{3}H]thymidine normally when washed and incubated in medium containing normal plasma. Both spontaneous incorporation and stimulated incorporation in response to various mitogens and antigens were inhibited. The inhibitory effect was identified with the chylomicron and very low density lipoprotein fractions isolated from plasma and was concentration-dependent. Lectin used to stimulate cultured cells and [^{3}H]thymidine used to measure responses were not bound to the lipoproteins in appreciable amounts. [^{3}H]-Thymidine incorporation correlated well with morphologic evidence of lymphoproliferation. The mechanism of the inhibitory effect of type IV or V hyperlipoproteinemic plasma upon the response tested was not identified but may be related to interaction between lipoproteins and the cell membranes. We suggest that these lipoproteins may also interfere with the function of other cells.

INTRODUCTION

Primary type IV hyperlipoproteinemia is characterized by elevated plasma concentrations of very low density lipoproteins (VLDL) [1] and is associated with an increased risk of atherosclerosis (1, 2). Type V hyperlipoproteinemia is characterized by elevated plasma levels of VLDL and chylomicrons and is associated with an increased risk of developing pancreatitis (3). An undue susceptibility to infections or manifestations of immunologic impairment have not been associated with either lipid transport disorder.

We wish to report that plasma from patients having a type IV or type V hyperlipoproteinemic pattern inhibits proliferation by cultured mononuclear leukocytes (MNL) when stimulated with mitogens or antigens. This inhibition was identified with both VLDL and chylomicrons; patient lymphocytes responded normally after they were washed and then cultured in medium containing normal plasma.

METHODS

10 patients with type IV and 10 patients with type V primary hyperlipoproteinemia (Table I) were studied. The type of hyperlipoproteinemia was determined by standard diagnostic criteria (4). Obesity, hypertension, and diabetes mellitus were present in several of these patients; atherosclerotic vascular disease was common among patients with a type IV pattern. These patients gave no history of unusual susceptibility to infections. A normal capacity for delayed cutaneous responses ($\geq$ 10 mm of induration) was established in patients skin-tested with *Candida albicans* (Hollister-Steir, Inc., Spokane Wash.) and with streptokinase-streptodornase (Lederle Laboratories, Div. of American Cyanamid Co., Pearl River, N. Y.) antigens. Healthy young male volunteers donated normal blood. All blood was collected in heparinized syringes. Normal plasma in most experiments was from one healthy individual (O. D. T.) and contained $\leq$ 170 mg/100 ml cholesterol and $\leq$ 80 mg/100 ml triglyceride; plasma from a second healthy young male with similar low concentrations of cholesterol

This material was presented in part at the Southern Section meeting of the American Federation for Clinical Research, 30 January 1975, New Orleans, La.

Received for publication 19 January 1976 and in revised form 10 June 1976.

[1] *Abbreviations used in this paper:* MNL, mononuclear leukocytes; MNL_x, irradiated mononuclear leukocytes; PHA, phytohemagglutinin-M; VLDL, very low density lipoproteins.

TABLE I
Clinical Data on Patients

	Type IV	Type V
Number of patients	10	10
Age (mean±SD)	45.5±8.7	46.9±8.7
Males:females	3:7	5:5
Patients with obesity	6	6
Patients with hypertension	4	6
Patients with diabetes mellitus	6	3
Patients with atherosclerotic vascular disease	4	1
Patients with delayed cutaneous responses/subjects tested	5/5	4/4
Plasma triglyceride, *mg/100 ml*		
Mean±SD*	571±186	2,609±1,179
Range	336–844	778–4,440
Plasma cholesterol, *mg/100 ml*		
Mean±SD	211±62	379±99
Range	105–312	220–487

* Normal values: triglyceride 10–190 mg/100 ml, cholesterol 120–265 mg/100 ml.

and triglyceride was used in a few experiments without appreciable change in results.

Plasma triglycerides and cholesterol were quantitated with an AA-2 AutoAnalyzer (5) (Technicon Instruments Corp., Tarrytown, N. Y.). Chylomicrons were separated under sterile conditions from patient plasma by centrifugation in cellulose nitrate tubes for 5.67×10^6 $g\cdot$min at 10°C in a Beckman L2-65B ultracentrifuge with a SW 50.1 swinging bucket rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) (6). The chylomicron layer was then transferred with a needle and syringe into polypropylene tubes. VLDL were similarly isolated from plasma by ultracentrifugation for 2.38×10^6 $g\cdot$h (7); chylomicrons were removed before VLDL from type V plasma. VLDL were separated from 500 ml of normal plasma, obtained by plasmapheresis from a single donor, concentrated with a membrane filter (Amicon Corp., Lexington, Mass.), and resuspended in fresh autologous plasma. This plasma concentrate of normal VLDL contained 1.008 g/100 ml triglycerides and 248 mg/100 ml cholesterol. Lipids were extracted from a mixture of chylomicrons and VLDL isolated from type V hyperlipoproteinemic plasma with chloroform-methanol (2:1) (8). Chylomicrons, VLDL, or extracted lipids were thoroughly mixed with normal plasma before addition to the culture medium.

MNL were separated from heparinized blood by floatation on Ficoll-Hypaque (9). Triplicate cultures were prepared in flat-bottomed microtiter plates (Linbro Chemical Co., New Haven, Conn.) and incubated for 7 days (except where specified otherwise). Our leukocyte culture technique, the preparation of mitogens and antigens, and the method employed to measure [^{3}H]thymidine incorporation have been described elsewhere in detail (10–12). Proliferative stimuli tested included phytohemagglutinin (PHA) (Difco Laboratories, Detroit, Mich.), pokeweed mitogen (Grand Island Biological Co., Grand Island, N. Y.), dialyzed *C. albicans* and freshly reconstituted streptokinase-streptodornase. Stimulation in the mixed leukocyte reaction was with 2×10^5 MNL-lymphocytes per culture shortly after exposure to 2,500 R of X-irradiation. [^{3}H]Thymidine incorporation in unstimulated cultures was recorded directly. Net incorporation was recorded in stimulated cultures after subtracting spontaneous incorporation in paired unstimulated cultures. Proliferative responses to both *C. albicans* and streptococcal antigens were measured and the higher net incorporation was recorded as the representative response to soluble antigens.

PHA was labeled with ^{125}I (Na ^{125}I, Amersham/Searle Corp., Arlington Heights, Ill., sp act approximately 14 mCi/μg I) by the chloramine T procedure (13). Labeled PHA was dialyzed against phosphate-buffered saline until the dialysis fluid was free of radioactivity. The dialyzed ^{125}I-PHA contained 1,269 cpm/μg protein, measured by the Lowry method.

RESULTS

Type V and type IV hyperlipoproteinemic plasma and [^{3}H]thymidine incorporation. In our culture system, plasma comprises 25% by volume of the medium. The effect of substituting allogeneic plasma for autologous plasma upon [^{3}H]thymidine incorporation was first evaluated with MNL and plasmas from healthy subjects. Incorporation was compared in unstimulated cultures and in cultures stimulated with PHA, allogeneic irradiated MNL (MNL$_x$), and soluble antigens. In 10 experiments, there was no significant difference in incorporation when MNL were cultured with autologous or allogeneic normal plasma.

Similar experiments were performed on 10 patients with a type V hyperlipoproteinemic pattern. Cultures were prepared concomitantly on patient MNL and MNL from a healthy subject. Paired cultures were set up on each cell preparation with patient plasma and with normal plasma in the medium. Brisk incorporation occurred in stimulated cultures of washed patient and normal MNL when cultured in medium that contained third-party normal plasma (Fig. 1). Incorporation was significantly less when the medium contained type V

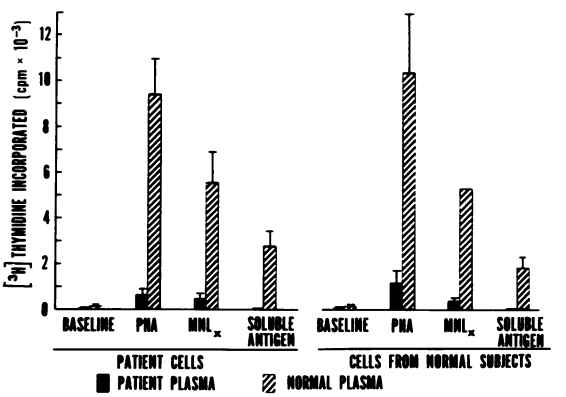

FIGURE 1 Comparison of [^{3}H]thymidine incorporation by MNL from patients with type V hyperlipoproteinemia and healthy subjects in culture medium containing type V hyperlipoproteinemic or normal plasma.

TABLE II

Inhibitory Effect of Patient Plasma and Lipoprotein Fractions Added to Normal Plasma upon Mixed Leukocyte Responses (Mean±SEM of Five Experiments)

Plasma in medium	Type IV plasma			Type V plasma		
	Plasma triglycerides	Mixed leukocyte response*	P‡	Plasma triglycerides	Mixed leukocyte response	P‡
	mg/100 ml	*cpm*		*mg/100 ml*	*cpm*	
Patient	670±86	14,200±6,100	<0.05	2,600±1,483	1,000±500	<0.01
Patient minus lipoproteins§	157±8	49,100±10,600		204±50	23,200±3,900	
Normal	64±6	86,400±8,300		72±4	49,100±11,200	
Normal plus lipoproteins§	1,270±249	3,400±1,600	<0.001	1,900±931	4,500±1,700	<0.05

* [^{3}H]thymidine incorporated.

‡ Student *t* test.

§ VLDL in type IV; chylomicrons and VLDL in type V.

hyperlipoproteinemic plasma ($P < 0.01$, Wilcoxon rank sum test). Incorporation in unstimulated cultures was also significantly less in medium with patient than with normal plasma ($P < 0.01$). Similar results were obtained with MNL and plasma from 10 patients with a type IV hyperlipoproteinemic pattern.

Lipoprotein concentration and [^{3}H]thymidine incorporation. [^{3}H]Thymidine incorporation by MNL from healthy subjects was evaluated in the MLR. Incorporation was compared in a medium that included (*a*) type IV or type V hyperlipoproteinemic plasma, (*b*) the same patient plasma from which most chylomicrons and/or VLDL had been removed, (*c*) normal plasma, or (*d*) the same normal plasma to which lipoprotein fractions isolated from the hyperlipoproteinemic plasma had been added. [^{3}H]Thymidine incorporation was significantly less when the culture medium contained whole patient plasma than when the medium contained the same plasma minus most of its chylomicrons and/or VLDL (Table II). Responses were significantly greater with normal plasma than with the same normal plasma plus chylomicrons and/or VLDL.

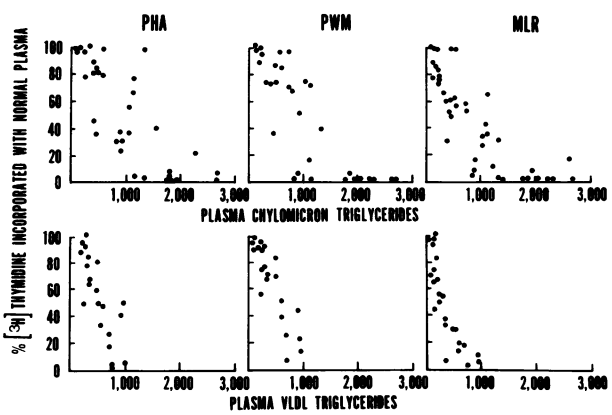

FIGURE 2 The relationship between concentration of VLDL or chylomicrons, in milligrams per 100 ml, added to culture media and [^{3}H]thymidine incorporation by MNL from healthy subjects. PWM, pokeweed mitogen; MLR, mixed leukocyte response.

Chylomicron and VLDL fractions isolated from patient plasma were added to normal plasma and lipoprotein concentrations varied by dilution with normal plasma. The relationship between triglyceride concentrations in test plasma preparations and [^{3}H]thymidine incorporation in response to PHA, PWM, and allogeneic MNL$_x$ stimulation is shown in Fig. 2. Responses are expressed as percent incorporation recorded by the same MNL when cultured with normal plasma. Incorporation was inversely related to triglyceride concentrations. At a triglyceride concentration of 1.008 g/100 ml, VLDL from normal plasma inhibited these responses like VLDL from patient plasma.

Inhibition of lymphoproliferation by VLDL and chylomicrons. Paired cultures of MNL from healthy subjects were incubated for 3 days in medium that included type V hyperlipoproteinemic or normal plasma. Responses to PHA stimulation measured by [^{3}H]thymidine incorporation and percentages of transformed lymphocytes were compared. Transformation as well as incorporation was significantly less with patient plasma than with normal plasma ($P < 0.01$). Hemocytometer white cell counts performed daily through 7 days of incubation were similar in unstimulated MNL cultures that contained patient or normal plasma. Lipid extracted from a VLDL-chylomicron mixture and added in high concentrations to medium did not inhibit mitogen- or antigen-stimulated [^{3}H]thymidine incorporation by normal MNL.

To evaluate the possibility of significant binding of PHA and [^{3}H]thymidine to the lipoproteins, medium was prepared in the usual fashion with type V hyperlipoproteinemic and with normal plasma. ^{125}I-PHA and [^{3}H]thymidine were added separately in proportions used in MNL cultures. After thorough mixing and 18 h of incubation, the lipoproteins were removed by ultra-

centrifugation and passed over an agarose gel column, and the radioactivity in the lipoprotein fraction was determined. The results indicated that less than 0.005% of the lectin and unappreciable amounts of [^{3}H]thymidine remained with the lipoproteins.

DISCUSSION

This study presents evidence that plasma from patients with type IV or V hyperlipoproteinemia decreases proliferation by cultured lymphocytes. The reduction of [^{3}H]thymidine incorporation by cultured MNL stimulated with mitogens or allogeneic leukocyte antigens was quantitatively related to the concentration of VLDL or chylomicrons added to the culture medium. Others have shown that [^{3}H]thymidine incorporation correlates with DNA synthesis by cultured lymphocytes (14). Low [^{3}H]thymidine incorporation in stimulated cultures that contained hyperlipoproteinemic plasma correlated with a reduction in lymphoblastogenesis in the present study. Additional experiments showed that the phenomenon observed was not due to shortened survival of cultured MNL or to complexing of PHA or [^{3}H]thymidine to lipoproteins. The most likely explanation for this new observation is that elevated concentrations of VLDL or chylomicrons in culture medium inhibit lymphoproliferation. Our data do not indicate whether these lipoproteins interfere with lymphocytes, macrophages, or both.

The mechanisms involved in the inhibition of lymphoproliferation by these lipoprotein fractions were not defined. Preliminary evidence indicates that ^{125}I-VLDL binds to the mononuclear cell, as has been observed elsewhere with cultured fibroblasts (15). Perhaps chylomicrons and VLDL inhibit lymphoproliferation by interfering with events at the cell membrane level, presumably by steric hindrance, preventing the normal interaction between stimulus and cell membrane. Alternatively, the composition and properties of the cell membrane may be altered in the presence of the hyperlipidemic plasma. It is also possible that these lipoproteins are internalized by cells (16), where they may alter metabolic processes. Studies are in progress to determine the mechanisms that produce the effects reported here and to determine whether the intact lipoprotein or the lipid or apoprotein constituents are responsible. The effect of VLDL or chylomicrons upon lymphoproliferation in vitro is reversible by washing patient MNL and then culturing them in medium that contains normal plasma.

Plasma from patients with other disorders has been reported to inhibit proliferative responses by cultured lymphocytes. These disorders include chronic mucocutaneous candidiasis (11, 17), solid tumors (18, 19), primary tumors of lymphoid tissues (20, 21), and systemic lupus erythematosus (22).

Proliferation by sensitized lymphocytes in response to antigens is a normal correlate of delayed hypersensitivity (23). Impaired lymphoproliferative responsiveness occurs frequently with various immunodeficiencies (21, 24–26). Abnormal plasmas with a type IV or V lipoprotein pattern may interfere with other immune responses as well, particularly those that involve lymphocyte proliferation (27). The concentration of plasma in our cultures is about half the normal concentration of plasma in the blood. Since inhibition of [^{3}H]thymidine incorporation by VLDL or chylomicrons is concentration dependent, plasma from patients with type IV or V hyperlipoproteinemia may be a more potent inhibitor of immune responses within the circulation than in our in vitro culture system.

Patients with primary type IV or V hyperlipoproteinemia are not clinically immunodeficient. Since lipoproteins are macromolecules (28), plasma lipoproteins may be largely confined within the vascular bed, analogous to macroglobulins (29). Immune reactive cells outside the circulatory bed may not be exposed to the high concentrations of lipoproteins present in plasma. The immunobiological significance of our findings may, therefore, relate only to the intravascular compartment.

The possibility of interference with normal immune mechanisms within the vascular bed is suggested by our data. Viral-induced changes in the smooth muscle of blood vessels may have a role in the atherogenic process (30, 31). Perhaps patients in whom the immune process within blood vessels is suppressed have greater susceptibility to viral-induced changes in the walls of arteries.

If lipoproteins interfere with interactions between proliferative stimuli and MNL membranes, it is also possible that lipoproteins interfere with other important events at the cell surface level (such as hormone bindings). Interference of this type may also involve other cells, such as those that comprise vascular endothelium. The consequences of such alterations of cell membrane function may be highly relevant to the pathogenesis of clinical disorders (such as atherosclerosis) associated with type IV or V primary hyperlipoproteinemia.

ACKNOWLEDGMENTS

The authors are grateful to Mrs. Helen Jamison and Phyllis Seate and Mr. Mauro Nava for their assistance.

This work was supported in part by National Heart and Lung Institute contract 71-2156, National Institutes of Health grant R-R-00350 and National Cancer Institute grant CA17/32.

REFERENCES

1. Carlson, L. A., and L. E. Böttiger. 1972. Ischaemic heart-disease in relation to fasting values of plasma triglycerides and cholesterol. Stockholm prospective study. *Lancet* **1**: 865–868.
2. Salel, A. F., K. Riggs, D. T. Mason, E. A. Amsterdam, and R. Zelis. 1974. The importance of type IV hyperlipoproteinemia as a predisposing factor in coronary artery disease. *Am. J. Med.* **57**: 897–903.

3. Fredrickson, D. S., and R. L. Levy. 1972. Familial hyperlipoproteinemia. *In* The Metabolic Basis of Inherited Diseases. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill Book Company, New York. 3rd edition. 545–614.
4. Beaumont, J. L., L. A. Carlson, G. R. Cooper, Z. Fejar, D. S. Fredrickson, and T. Strasser. 1970. Classification of hyperlipidaemias and hyperlipoproteinaemias. *Bull. W. H. O.* **43**: 891–915.
5. Lipids and Lipoprotein Analysis. 1974. The Manual of Lab. Operation. The Lipid Research Clinic Program, I. Department of Health, Education, and Welfare Publication 75-628.
6. Skipski, V. P. 1972. Lipid composition of lipoproteins in normal and diseased states. *In* Blood Lipids and Lipoproteins, Quantitation, Composition and Metabolism. G. J. Nelson, editor. John Wiley & Sons, Inc., New York. 488.
7. Brown, W. V., R. I. Levy, and D. S. Fredrickson. 1969. Studies of the proteins in human plasma very low density lipoproteins. *J. Biol. Chem.* **244**: 5687–5694.
8. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**: 497–509.
9. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand. J. Clin. Lab. Invest. Suppl.* **97**: 77–89.
10. Twomey, J. J., O. Sharkey, Jr., J. A. Brown, A. H. Laughter, and P. H. Jordan, Jr. 1970. Cellular requirements for the mitotic response in allogeneic mixed leukocyte cultures. *J. Immunol.* **104**: 845–853.
11. Twomey, J. J., C. C. Waddell, S. Krantz, R. O'Reilly, P. l'Esperance, and R. A. Good. 1975. Chronic mucocutaneous candidiasis with macrophage dysfunction, a plasma inhibitor, and co-existent aplastic anemia. *J. Lab. Clin. Med.* **85**: 968–977.
12. Twomey, J. J., A. H. Laughter, S. Farrow, and C. C. Douglass. 1975. Hodgkin's disease. An immunodepleting and immunosuppressive disorder. *J. Clin. Invest.* **56**: 467–475.
13. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131-labelled human growth hormone of high specific activity. *Nature (Lond.).* **194**: 495–496.
14. Schellekens, P. T. A., V. P. Eijsvoogel. 1968. Lymphocyte transformation *in vitro*. Tissue culture conditions and quantitative measurements. *Clin. Exp. Immunol.* **3**: 571–584.
15. Brown, M. S., and J. L. Goldstein. 1974. Familial hypercholesterolemia: defective binding of lipoproteins to cultured fibroblasts associated with impaired regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. *Proc. Natl. Acad. Sci. U. S. A.* **71**: 788–792.
16. Steinberg, D., and P. J. Nestel. 1975. Interactions of human skin fibroblasts with low density lipoproteins (LDL) covalently bonded to sepharose beads. *Circ. Suppl.* II, **52(II)**: II–59. (Abstr.)
17. Canales, L., R. O. Middlemas, III, J. M. Louro, and M. A. South. 1969. Immunological observations in chronic mucocutaneous candidiasis. *Lancet.* **2**: 567–571.
18. Silk, M. 1967. Effect of plasma from patients with carcinoma on in vitro lymphocyte transformation. *Cancer.* **20**: 2088–2089.
19. Gatti, R. A., D. B. Garrioch, and R. A. Good. 1970. Depressed PHA responses in patients with non-lymphoid malignancies. *Proc. Leucocyte Cult. Conf.* **5**: 339–358.
20. Gaines, J. D., M. A. Gilmer, and J. S. Remington. 1972. Deficiency of lymphocyte antigen recognition in Hodgkin's disease. *Natl. Cancer Inst. Monogr.* **36**: 117–121.
21. Twomey, J. J., A. H. Laughter, S. Lazar, and C. C. Douglas. 1975. Reactivity of lymphocytes from primary neoplasms of lymphoid tissues. *Cancer.* In press.
22. Wernet, P., and H. G. Kunkel. 1973. Antibodies to a specific surface antigen of T cells in human sera inhibiting mixed leukocyte culture reactions. *J. Exp. Med.* **138**: 1021–1026.
23. Kerby, G. R. 1968. Correlation of tuberculin skin reaction with in vitro lymphocyte transformation. *Am. Rev. Respir. Dis.* **97**: 904–908.
24. Kirschhorn, K., R. R. Schreibman, F. H. Bach, and L. E. Siltzbach. 1964. In vitro studies of lymphocytes from patients with sarcoidosis and lymphoproliferative diseases. *Lancet.* **2**: 842–843.
25. Han, S. H., R. S. Weiser, and Y. C. Lin. 1971. Transformation of leprous lymphocytes by leprolin, tuberculin and phytohemagglutinin. *Int. J. Lepr.* **39**: 789–795.
26. Dupont, B., and R. A. Good. 1975. Lymphocyte transformation in vitro in patients with immunodeficiency diseases: use in diagnosis, histocompatiblity testing and monitoring treatment. *Birth Defects: Orig. Artic. Ser.* **1**: 477–485.
27. Rocklin, R. E., R. P. MacDermott, L. Chess, S. F. Schlossman, and J. R. David. 1974. Studies on mediator production by highly purified human T and B lymphocytes. *J. Exp. Med.* **140**: 1303–1316.
28. Fredrickson, D. S., A. M. Gotto, and R. I. Levy. 1972. Familial lipoprotein deficiency (Abetalipoproteinemia, hypobetalipoproteinemia, and Tangier disease). *In* The Metabolic Basis of Inherited Diseases. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill Book Company, New York. 3rd edition. 493–530.
29. Bloch, K. J., and D. G. Maki. 1973. Hyperviscosity syndromes associated with immunoglobulin abnormalities. *Semin. Hematol.* **10**: 113–124.
30. Benditt, E. P., and J. P. Benditt. 1973. Evidence for a monoclonal origin of human atherosclerotic plaques. *Proc. Natl. Acad. Sci. U. S. A.* **70**: 1753–1756.
31. Burch, G. E. 1974. Viruses and arteriosclerosis. *Am. Heart J.* **87**: 407–412.