Increased Superoxide Production by Mononuclear Cells of Patients With Hypertriglyceridemia and Diabetes

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Diabetic patients with hypertriglyceridemia frequently develop atherosclerosis. Because superoxide (O_2^-) is suspected to play an important role in the initiation of atherosclerosis, we investigated whether an abnormal amount of O_2^- was produced by circulating mononuclear cells of patients with both diabetes mellitus and hypertriglyceridemia. The rate of production of superoxide dismutase–inhibitable O_2^- was measured when cells were stimulated by either 4β-phorbol 12β-myristate 13α-acetate (PMA) or by opsonized zymosan (OZ). In addition, the rates of O_2^- production by mononuclear cells drawn from three other groups (normal, solely diabetic, and solely hypertriglyceridemic) were determined. We found that the rate of O_2^- production by mononuclear cells from the diabetic hypertriglyceridemic group was significantly higher than that from normal, diabetic, and hypertriglyceridemic groups. When the rates of O_2^- production by mononuclear cells were plotted against the levels of plasma triglyceride for all individuals tested, they correlated positively ($r = .73$ in PMA stimulation and $r = .79$ in OZ stimulation, $P < .01$). However, the rate of O_2^- production did not relate to other parameters, i.e., plasma cholesterol level, hemoglobin A_1 level in erythrocytes, and the molar ratio of free cholesterol to phospholipid in mononuclear cells. Thus, we concluded that the observed elevated rate of O_2^- production in the diabetic hypertriglyceridemic mononuclear cells was a reflection of a hypertriglyceridemic condition and was not unique to the diabetic hypertriglyceridemic condition. Also, O_2^- may be involved in the pathogenesis of atherosclerosis in diabetic hypertriglyceridemic patients when atherogenic factors specific to diabetes are concomitantly present.
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Patients with both diabetes mellitus and hypertriglyceridemia are more prone to develop atherosclerosis than patients with either disease (1,2). Thus, an atherogenic condition unique to diabetic hypertriglyceridemic patients may be hypothesized. The condition may be established when one of two situations exists: *1*) an atherogenic factor(s) found only in the diabetic hypertriglyceridemic patients is (are) present, or *2*) atherogenesis is accelerated when atherogenic factors associated with hypertriglyceridemia and diabetes are combined. In search of the factor(s) in these patients, we examined various parameters associated with the development of atherosclerosis. In our previous investigations of these patients, we found that factors impairing the catabolism of low-density lipoprotein (LDL) and prolonging the half-life of LDL in serum of these patients were associated with hypertriglyceridemia and were not unique to the combination of diabetes and hypertriglyceridemia (3). Because oxidized LDL by free radicals released from monocytes promotes the development of atherosclerosis (4), there is a possibility that monocytes of the patients with both diabetes and hypertriglyceridemia produce more free radicals than those with only hypertriglyceridemia. To examine this possibility, we compared the rates of superoxide (O_2^-) production by mononuclear cells from diabetic hypertriglyceridemic patients with those of diabetic, hypertriglyceridemic, and healthy individuals.

MATERIALS AND METHODS

Subjects. Four groups of subjects were studied as follows: individuals who had neither diabetes mellitus nor hypertriglyceridemia (normal group); individuals who had diabetes mellitus but not hypertriglyceridemia (diabetic group); individuals who had hypertriglyceridemia but not diabetes mellitus (hypertriglyceridemic group); and individuals who had both diabetes mellitus and hypertriglyceridemia (diabetic hypertriglyceridemic group). Patients with alcoholism, uremia, estrogen supplementation, or hypothyroidism were excluded from this study. Non-insulin-dependent diabetes

mellitus (NIDDM) was diagnosed with the criteria from the National Diabetes Data Group (diabetes mellitus was diagnosed on the basis of the 75-g oral glucose tolerance test; 5). Insulin-dependent diabetes mellitus (IDDM) was diagnosed if the onset of diabetes was sudden and ketosis prone and positive islet antibodies were present. With diabetic patients, the blood glucose was controlled without changes in treatment for 4 mo before the experiments and was stable at the time of blood withdrawal. Hypertriglyceridemia was diagnosed when the fasting plasma triglyceride levels were >150 mg/dl on three occasions. The 75-g oral glucose tolerance test was also performed on all patients with hypertriglyceridemia.

The normal group consisted of 18 subjects (7 men and 11 women, ages 38–60 yr). The diabetic group consisted of 10 subjects (5 men and 5 women, ages 37–72 yr). Nine had NIDDM, and 1 had IDDM; 4 were being treated with insulin, 5 with oral hypoglycemic agents, and 1 with diet alone. The hypertriglyceridemic group included 10 patients (6 men and 4 women, ages 40–62 yr). The diabetic hypertriglyceridemic group comprised 13 patients (7 men and 6 women, ages 53–65 yr). All of these had NIDDM. One was being treated with insulin, 8 were being treated with oral hypoglycemic agents, and 4 were being treated by diet alone. The age and sex distribution was matched among the four groups. There was no major difference in the medications for diabetes between the diabetic group and the diabetic hypertriglyceridemic group. At the time of blood collection, none of the subjects had evidence of acute infection or other inflammatory processes.

Preparation of cells. Mixed mononuclear cells of blood samples from fasting subjects were separated by density gradient centrifugation according to the method of Böyum (6). Blood was collected into syringes containing heparin to give a final concentration of 10 U/ml. Heparinized blood was layered over a Ficoll-Paque gradient (Pharmacia, Piscataway, NJ) and was centrifuged for 30 min at 500 × *g* at 23°C. The mixed mononuclear cell band was removed by aspiration with a siliconized glass Pasteur pipette, and the cells were washed twice in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS). The number of monocytes in the mononuclear cell population was verified on the basis of analysis of volume distribution by a Coulter Channelyzer (Hialeah, FL). The mean ± SE percent of monocytes in the 51 mononuclear cell samples was 32.4 ± 3.4%. Identity of monocytes was confirmed by nonspecific-esterase staining. The percent of cells stained was equivalent to the result obtained by the Coulter Channelyzer. The percent of neutrophil contamination was <1%. The cells were resuspended in HBSS containing 2 mM glucose and 1 mM $CaCl_2$ to give a final count of 3 × 10^6 monocytes/ml. The tubes containing cell suspensions were kept on ice until O_2^- release was determined.

O_2^- release from stimulated mononuclear cells. O_2^- production was measured as superoxide dismutase (SOD)–inhibitable reduction of ferricytochrome *c* (7), with 4β-phorbol 12β-myristate 13α-acetate (PMA) or opsonized zymosan (OZ) as a stimulant. O_2^- measurement was performed on the samples of six to eight subjects during each of the 8 days of the experiment. O_2^- release by PMA was examined in all 51 samples, and 23 of these samples were also examined in parallel for O_2^- release by OZ. To prepare OZ, zymosan particles were washed twice in phosphate-buffered saline (PBS), and 10 ml of AB^- serum was added to 100 mg of the zymosan pellet. The mixture was whirled with a Vortex (Scientific Industries, Bohemia, NY) and rotated for 1 h at 37°C. After centrifugation, the serum was removed, and the particles were washed with PBS. The zymosan particles were resuspended in HBSS containing 2 mM glucose and 1 mM $CaCl_2$ at a concentration of 10 mg/ml. Each 5-ml reaction mixture contained 5 × 10^5 monocytes, 100 μM cytochrome *c*, 2 mM glucose, and 1 mM $CaCl_2$ in HBSS. Paired experiments with and without 10 μg/ml SOD were performed. PMA or OZ was added at a concentration of 1 μg/ml or 1.1 mg/ml, respectively. The reaction mixtures were incubated at 37°C in a water bath for 20 min. At the end of the incubation, the tubes were placed on ice and then centrifuged at 900 × *g* for 5 min at 4°C to remove the cells. The supernatant was measured spectrophotometrically at 550 nm. The reduced cytochrome *c* (in nanomoles) was determined from the increase in the absorbance at 550 nm: E = 21.0 cm^{-1} mM^{-1}, where E is the extinction coefficient (8). The results were expressed as nmol · 10^{-6} monocytes · 20 min^{-1}. All procedures, from the time blood was drawn until O_2^- release was measured, were performed within 5 h. The time taken for these experiments was approximately the same for all 8 days. Values for day-to-day variation were minimal. For example, the mean ± SD of PMA-stimulated O_2^- released from 10^6 mononuclear cells of a healthy subject in 4 days was 3.5 ± 0.4 nmol/20 min.

Molar ratio of free cholesterol to phospholipid in mononuclear cells. The molar ratio of free cholesterol to phospholipid was determined when adequate cells were available for analysis (22 subjects). The molar ratio of free cholesterol to phospholipid was determined with thin-layer chromatography with a flame ionization detector (9). The number of mononuclear cells for each sample was adjusted to 5 × 10^6 cells constituting 31.7 ± 4.7% monocytes. Lipid

TABLE 1
Plasma glucose, HbA_1, and lipid concentration in normal, diabetic, diabetic hypertriglyceridemic, and hypertriglyceridemic subjects

Group	*n*	Glucose (mg/dl)	HbA_1 (%)	Triglyceride (mg/dl)	Cholesterol (mg/dl)
Nondiabetic	18	84 ± 10 (76–102)	ND	99 ± 36 (52–141)	208 ± 29 (158–217)
Diabetic	10	165 ± 50 (126–238)	8.4 ± 1.8 (5.5–12.5)	111 ± 21 (81–138)	211 ± 10 (184–216)
Hypertriglyceridemic	10	84 ± 8 (72–97)	ND	352 ± 110 (230–538)	235 ± 47 (171–266)
Diabetic hypertriglyceridemic	13	176 ± 81 (105–346)	8.8 ± 2.2 (5.9–11.5)	427 ± 264 (229–1209)	226 ± 45 (161–274)

Values are means ± SD with ranges in parentheses.
Normal range of hemoglobin A_1 (HbA_1) is 5.5–8.0%.
ND, not determined.

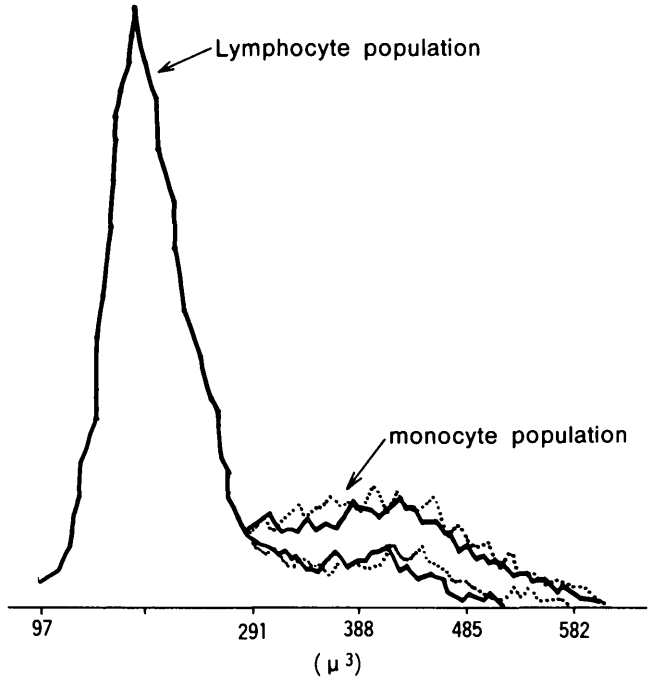

FIG. 1. Distribution curves of mononuclear cell volume. Size distribution information from Channelyzer was plotted on X-Y recorder. *Solid lines*, monocyte population from 28 normolipidemic subjects; *dotted lines*, from 23 hypertriglyceridemic subjects.

was extracted from mononuclear cells by the method of Folch et al. (10), with 10 mg/dl cholesterol acetate as an internal standard. The lipid extract was applied to a Chromarod and developed in a mixture of *n*-hexane–diethyl ether (9:1 vol/vol) for 40 min at 20°C. The Chromarod was passed through a flame ionization detector after drying for 3 min at 110°C. Each fraction of cholesterol, cholesterol acetate, triglyceride, free cholesterol, and phospholipid was detected by the flame ionization detector. The amount of free cholesterol and phospholipid was calculated from the standard curves, and the molar ratio of free cholesterol to phospholipid was determined.

Separation of very-low-density lipoprotein (VLDL). After patients fasted overnight, 50 ml blood was drawn into tubes containing EDTA (1 mg/ml). VLDL (density <1.006 g/ml) was separated by ultracentrifugation (11). The concentration of protein in VLDL was measured by the method of Lowry et al. (12).

Other determinations. Plasma glucose, triglyceride, cholesterol, and hemoglobin A_1 were analyzed as routine samples in the central clinical laboratory of Tokai University Hospital.

Statistical methods. Comparisons between the groups were made by Student's *t* test (13). All values were expressed as means ± SD. Correlation coefficients were determined by the standard statistical method (14).

RESULTS

The diabetic and diabetic hypertriglyceridemic groups had higher fasting plasma glucose levels accompanied by high levels of hemoglobin A_1 than the normal and hypertriglyceridemic groups (Table 1). The mean plasma triglyceride levels in the hypertriglyceridemic and diabetic hypertriglyceridemic groups were approximately fivefold higher than in the normal and diabetic groups. The mean value of triglyceride of the diabetic hypertriglyceridemic group was higher than that of the hypertriglyceridemic group (Table 1). The volume distribution of monocytes analyzed by Coulter Channelyzer is shown in Fig. 1. The pattern of volume distribution of cells from the hypertriglyceridemic group was similar to that from the normal group.

There was a significant positive correlation between the amount of O_2^- released by mononuclear cells stimulated by PMA and that by OZ (Fig. 2), but the amount released by mononuclear cells when stimulated by PMA was greater than that stimulated by OZ in all four groups (Table 2). With the PMA stimulation, the rate of O_2^- production by the diabetic hypertriglyceridemic mononuclear cells (28.1 ± 7.2

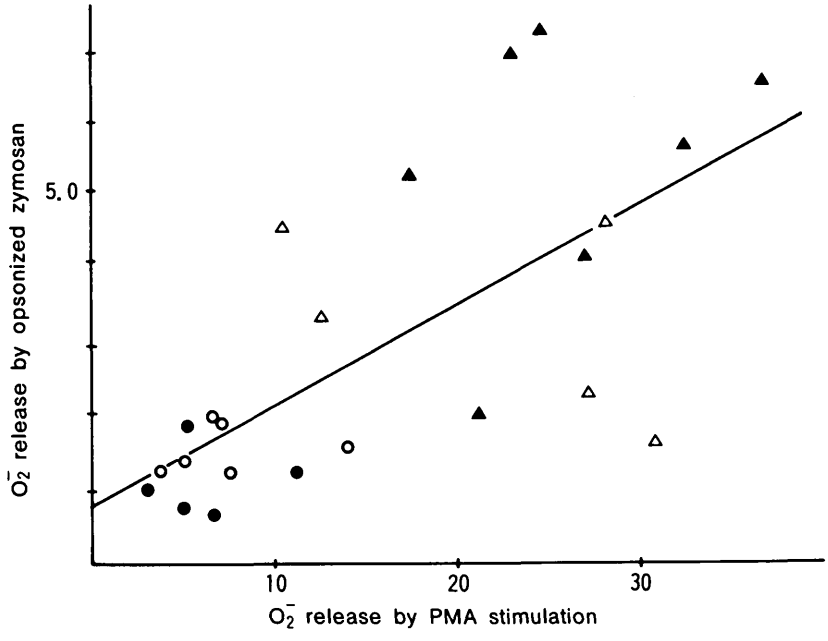

FIG. 2. Correlation between superoxide (O_2^-) released by 4β-phorbol 12β-myristate 13α-acetate (PMA) stimulation (abscissa) and O_2^- released by opsonized zymosan from 4 groups (○, normal subjects; ●, diabetic subjects; ▲, diabetic hypertriglyceridemic subjects; △, hypertriglyceridemic subjects). $r = .68$, $P < .01$.

TABLE 2
Superoxide release from mononuclear cells

Group	O_2^- release by PMA (nmol · 10^{-6} cells · 20 min^{-1})	O_2^- release by opsonized zymosan (nmol · 10^{-6} cells · 20 min^{-1})
Nondiabetic	8.9 ± 3.2 (18)	1.5 ± 0.3 (6)
Diabetic	7.5 ± 3.9 (10)	1.1 ± 0.5 (5)
Hypertriglyceridemic	21.3 ± 7.6 (10)*	3.3 ± 1.4 (5)†‡
Diabetic hypertriglyceridemic	28.1 ± 7.2 (13)*§	5.4 ± 1.8 (7)*§

Values are means ± SD with number of subjects in parentheses. O_2^-, superoxide; PMA, 4β-phorbol 12β-myristate 13α-acetate.
*$P < .001$ vs. nondiabetic and diabetic groups.
†$P < .02$ vs. diabetic group.
‡$P < .05$ vs. nondiabetic group.
§$P < .05$ vs. hypertriglyceridemic group.

nmol · 10^{-6} cells · 20 min^{-1}) was the highest, followed in order by the hypertriglyceridemic mononuclear cells (21.3 ± 7.6 nmol · 10^{-6} cells · 20 min^{-1}), the normal mononuclear cells (8.9 ± 3.2 nmol · 10^{-6} cells · 20 min^{-1}), and the diabetic mononuclear cells (7.5 ± 3.9 nmol · 10^{-6} cells · 20 min^{-1}). Statistical analysis of the data revealed that the rates of O_2^- production between the diabetic hypertriglyceridemic and normal cells, diabetic hypertriglyceridemic and diabetic cells, hypertriglyceridemic and normal cells, and hypertriglyceridemic and diabetic cells were significantly different. However, no significant difference in O_2^- production rates was observed between the cells from diabetic patients and those from normal individuals. The differences and similarities were similar when the cells were stimulated by OZ (Table 2).

Because both the diabetic hypertriglyceridemic and hypertriglyceridemic cells had extremely elevated levels of O_2^- production, individual O_2^- production rates were plotted against individual serum level of triglyceride. It was found that the rates of O_2^- production correlated significantly with the serum level of triglyceride (Fig. 3). No correlation was observed between the released O_2^- and the levels of plasma cholesterol, fasting blood glucose, or hemoglobin A_1.

To examine the effect of VLDL on the O_2^- release in vitro, the O_2^- release from mononuclear cells of the normal group was determined after addition of VLDL (150 and 300 mg/dl). The amount of O_2^- released was not affected by the change in triglyceride concentration.

To evaluate whether the lipid composition of the cell membrane of mononuclear cells had influenced the amount of O_2^- released, the molar ratio of free cholesterol to phospholipid was measured in the lipid extract of mononuclear cells from the four groups. The molar ratio of free cholesterol to phospholipid tended to be higher in the hypertriglyceridemic and diabetic hypertriglyceridemic groups compared with the diabetic and normal groups. However, the differences were not statistically significant (Table 3). The molar ratio of free cholesterol to phospholipid did not relate to plasma triglyceride concentration, plasma cholesterol concentration, or the amount of O_2^- released by mononuclear cells.

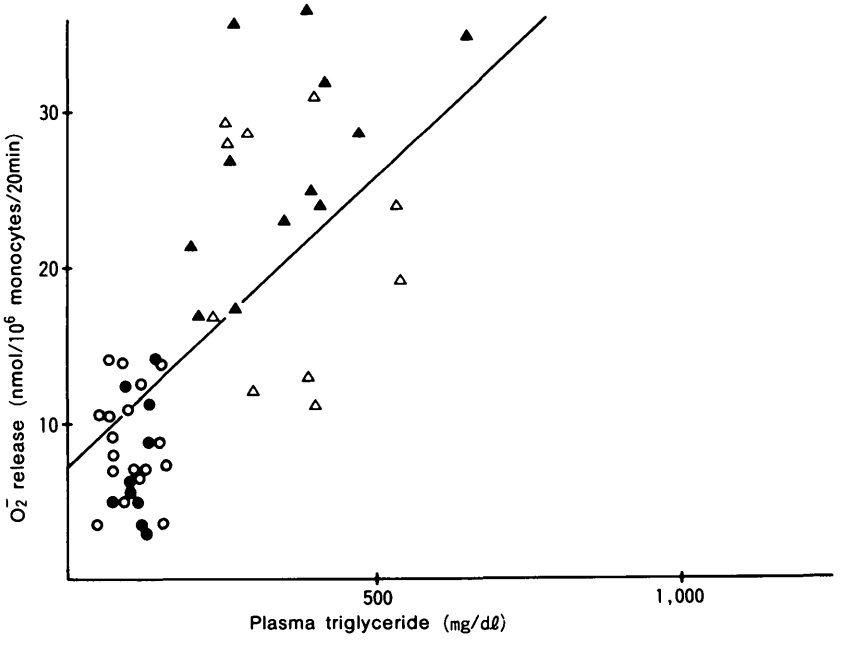

FIG. 3. Correlation between 4β-phorbol 12β-myristate 13α-acetate–stimulated superoxide (O_2^-) release and plasma triglyceride level from 4 groups (○, normal subjects; ●, diabetic subjects; ▲, diabetic hypertriglyceridemic subjects; △, hypertriglyceridemic subjects). $r = .73$, $P < .01$.

TABLE 3
Molar ratio of free cholesterol to phospholipid in mononuclear cells

Group	*n*	Free cholesterol/phospholipid
Nondiabetic	9	0.34 ± 0.08
Diabetic	4	0.35 ± 0.04
Hypertriglyceridemic	4	0.41 ± 0.07
Diabetic hypertriglyceridemic	5	0.43 ± 0.09

Values are means ± SD.

DISCUSSION

In this investigation, we hypothesized that the O_2^- production by mononuclear cells from patients with both diabetes mellitus and hypertriglyceridemia is elevated and that O_2^- is primarily responsible for the onset of atherosclerosis in these patients. We found that the rate of O_2^- production by the diabetic hypertriglyceridemic mononuclear cells was the highest followed in order by the hypertriglyceridemic mononuclear cells, the normal mononuclear cells, and the diabetic mononuclear cells. The rates of O_2^- production by the diabetic hypertriglyceridemic and the hyperglyceridemic groups were significantly higher than those of the normal and diabetic groups. The amount released from the cells of diabetic hypertriglyceridemic subjects was also significantly higher than in the hypertriglyceridemic group. However, there was no significant difference between the normal and diabetic groups, which confirms the observation made by Kitahara et al. (15) that monocytes from diabetic patients whose blood glucose level was controlled and monocytes from healthy individuals both produced a similar amount of O_2^-.

The significant positive correlation between O_2^- released and the plasma triglyceride levels (Fig. 3) suggested that when the serum level of triglyceride was high, the O_2^- production by mononuclear cells was also high, regardless of whether the mononuclear cells were taken from subjects with or without diabetes. Consequently, the significant elevation in O_2^- produced by diabetic hypertriglyceridemic cells compared with that by hypertriglyceridemic cells is probably a reflection of the level of triglyceride in these sera, because the average level of triglyceride in sera of diabetic hypertriglyceridemic patients was higher than that of hypertriglyceridemic patients. Hence, with these results the proposed hypothesis must be rejected.

The rate of O_2^- production varies considerably among populations of circulating cells. As we report for the mononuclear cells, Ludwig et al. (16) have also reported that polymorphonuclear cells (PMN) from subjects with hyperlipoproteinemia had enhanced O_2^- release compared with healthy control subjects. Our preliminary studies also indicated the PMN, but not lymphocytes, from diabetic hypertriglyceridemic and hypertriglyceridemic patients had elevated levels of O_2^- production (data not shown). These findings suggested that when the serum level of triglyceride is high, the circulating leukocytes produce more O_2^-. In this regard, it remains to be determined whether cells with a higher capacity to produce O_2^- are selectively released into the circulation from bone marrow or whether the circulating cells produce more O_2^- in response to the hypertriglyceridemic environment.

The monocyte population is heterogenous (17), and the larger monocytes supposedly produce more O_2^- than the smaller monocytes. When the size of monocytes from hypertriglyceridemic patients and from healthy individuals were compared, however, we found no difference (Fig. 1). This finding suggests that there is no gross morphological difference between these mononuclear cell popoulations. In search of some minor difference, the lipid composition of plasma membrane of hypertriglyceridemic mononuclear cells was compared with that from healthy individuals. We considered this analysis worthwhile because a high ratio of free cholesterol to phospholipid results in hyperaggregation of platelets (18) and abnormal erythrocytes (19). When the molar ratio of free cholesterol to phospholipid was determined, no definite difference was observed in the hypertriglyceridemic group compared with the normal or diabetic groups in this study (Table 3). These observations suggest that there is no clear difference between the circulating mononuclear cells from hypertriglyceridemic patients and the cells from healthy individuals for the parameters examined.

There are reports that VLDL alters the activity of some membrane-associated enzymes, e.g., adenylate cyclase (20), and that an enzyme system that catalyzes respiratory burst is found in the cell membrane (21). Mononuclear cells from healthy individuals were exposed to VLDL in vitro to examine whether a high level of triglyceride would directly stimulate circulating mononuclear cells to produce more O_2^-. We found that the addition of VLDL failed to increase the rate of O_2^- production, as was found when PMN cells were incubated in 10% hyperlipoproteinemic serum (16). Although the parameters examined in this series of experiments were somewhat limited, we were unable to define when, where, or why the leukocytes of hypertriglyceridemic patients with or without diabetes acquire the enhanced ability to produce O_2^-.

The facts that the rate of O_2^- production by mononuclear cells is elevated when the serum triglyceride is elevated and that the incidence of atherosclerosis is high in well-controlled diabetic hypertriglyceridemic patients lead to some interesting speculations. Many recognized atherogenic factors have been associated with both uncontrolled and controlled diabetes mellitus; e.g., increased O_2^- production by monocytes (15), enhanced aggregation of platelets (22,23), and increased adhesion of erythrocytes (24) have been associated with poorly controlled diabetes, and hyperinsulinemia, consequent to the control of the diabetic state, may itself be atherogenic (25) and induce hyperproliferation of arterial smooth muscle cells (26) despite well-controlled diabetes. Hence, regardless of whether the diabetes is controlled, these patients have a tendency to develop atherosclerosis. Hypertriglyceridemia is also expected to be atherogenic. The hypertriglyceridemia has been reported to induce the formation of the foam cell, which is the major cell type found in the atherosclerotic lesion (27), and to prolong the half-life of LDL in plasma by impairing the catabolism of LDL (3). Because the level of O_2^- is already high in these plasma, the prolonged half-life will increase the chance for the LDL to be further oxidized by O_2^- (4). The oxidized LDL is considered to be a major atherogenic factor (28,29). Thus, we suggest that the effects of the atherogenic factors by dia-

betes mellitus and that by hypertriglyceridemia are additive in the induction of atherosclerosis. When the sum of the factors reaches a given level, atherosclerosis will result, such as when diabetes mellitus is accompanied by hypertriglyceridemia, even though each of the factors is itself insufficient to induce atherosclerosis.

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