Inhibition of In Vitro Lymphocyte Response by Streptozotocin-induced Diabetic Rat Serum

A Function of Very-Low-Density Lipoproteins

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SUMMARY

Immune function is generally reported to be impaired in uncontrolled diabetes mellitus. We have previously reported that very-low-density lipoproteins (VLDL) from diabetic rat serum injure endothelial cells in vitro. Thus, we wanted to determine if VLDL would also injure lymphocytes and impair the immunity of the streptozotocin-induced diabetic rat. The immunologic functions of spleen cells from normal and diabetic rats were studied using the in vitro mitogenic stimulation assay and mixed lymphocyte responses (MLR). The effects of diabetic serum and VLDL isolated from diabetic rat serum on normal spleen cells were also determined. Both the absolute peripheral blood leukocyte (PBL) and spleen cell numbers of diabetic rats were significantly decreased from those of normal rats ($P < 0.02$). In PBL there was a decreased percentage of lymphocytes and increased percentage of neutrophils (both $P < 0.02$). The mitogenic stimulation responses of spleen cells from diabetic rats were consistently lower than normal for 3 of the 4 mitogens used. The responses of con A-stimulated lymphocytes from diabetic spleens were significantly different ($P < 0.025$) from normal spleen cells. The MLR appeared to be normal. The decreased mitogenic responses of cells from diabetic spleens were not due to the induction of suppressor cells. Diabetic rat serum inhibited the proliferation of both control and mitogen-stimulated normal rat lymphocytes. Addition of increasing amounts of VLDL, isolated from diabetic serum, to normal spleen cell cultures also resulted in an exponential decrease in ^{3}H-thymidine incorporation. Complete inhibition of the response of normal spleen cells to PHA was achieved at a VLDL triglyceride concentration of 262 μg/ml. The 4-day-old lymphocyte cultures incubated with VLDL at 262-μg/ml concentration were only 11% viable, which was significantly lower than the 58% viability of control cultures without VLDL ($P < 0.005$). Removal of VLDL from diabetic rat serum eliminated the toxic effect of diabetic serum on spleen cell cultures. Reconstitution of the infranatant fraction ($d > 1.006$) with the VLDL fraction ($d < 1.006$) restored the toxic activity. These results indicate that the inhibitory effect of diabetic rat serum on the mitogenic responses of normal rat spleen cells is due to the toxicity of VLDL. **DIABETES *31*:1098–1104, December 1982.**

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The incidence of infection between well-controlled diabetics without vascular insufficiency and normal individuals appears to be similar.[1–4] However, both the severity and frequency of infection increase in uncompensated diabetes mellitus.[5] A decrease in cell-mediated immunity has been postulated to account for the increased incidence of infection in poorly controlled diabetics[6–10] and in one study, the lymphocyte transformation response correlated with the level of fasting hyperglycemia.[11]

We have recently shown that elevated levels of very-low-density lipoproteins (VLDL) from streptozotocin-induced diabetic rat serum are toxic to porcine aortic endothelial cells in vitro.[12] In this study the immunologic functions of spleen cells from diabetic rats were studied using the in vitro mitogenic stimulation assay and mixed lymphocyte responses in an attempt to determine if elevated levels of VLDL were also toxic to lymphocytes. We have shown that both the absolute peripheral blood leukocyte and spleen cell numbers of streptozotocin-induced diabetic rats were significantly decreased from those of normal rats. In diabetic rats, the percentage of peripheral blood lymphocytes was decreased significantly, while the percentage of neutrophils was significantly increased. The mitogenic stimulation responses of spleen cells from diabetic rats were consistently lower than those from normal rats and were significantly different when con A was used. This decreased response was not due to the existence of suppressor cells. Diabetic rat serum suppressed proliferation of both control and mitogen-stimulated lymphocyte cultures. Purified VLDL alone could account for the decreased ^{3}H-thymidine incorporation.

MATERIALS AND METHODS

Animals, sera, and VLDL preparation. Both male Wistar and Sprague-Dawley rats (Camm, Wayne, New Jersey) weighing approximately 200 g were used. Streptozotocin (100 mg/kg) in acidified saline was injected into the tail vein of rats fasted 18 h.[13] After 7 days the rats were killed by decapitation; the blood was collected and allowed to clot at 37°C for 1 h before centrifugation at 300 × g for 10 min at 4°C.

Diabetic serum used to prepare VLDL was centrifuged at 175,000 × g for 18 h at 4°C in a SW-41 rotor in a Beckman L5-75B ultracentrifuge (Beckman Instruments, Inc., Irvine, California).[14] The top VLDL fraction ($d < 1.006$) was removed using a tube slicer, diluted with cold 0.15 M NaCl, and recentrifuged under the above conditions.

VLDL were electrophoresed in 0.8% agarose gel[15] and stained with oil red O to determine the purity of this fraction. Normal rat serum was used as the standard. Triglycerides[16] were assayed on a Sequential Multiple Analyzer 18 (Technicon, Tarrytown, New York).

Peripheral blood leukocyte (PBL) and spleen cell preparations. Peripheral blood was collected from the trunk of decapitated normal or diabetic rats and mixed with phenol-free heparin (100 U/ml final concentration, A. H. Robins, Richmond, Virginia). One hundred microliters of blood was then mixed with 18 ml of Isoton II (Curtin Matheson Scientific, Inc., Houston, Texas) and 2 ml of Hematall (Fisher Scientific Co., Fairlawn, New Jersey), and the leukocytes counted in a Z_{BI} Coulter Counter (Hialeah, Florida). Blood smears were stained with Wright's stain for differential counting.

Spleens used for cell preparation were aseptically removed and gently teased in RPMI 1630 media (Gibco, Grand Island, New York). After filtration through gauze, the spleen cells were washed three times by centrifugation and counted on a hemacytometer. The viability of the spleen cell preparations as determined by trypan blue exclusion ranged from 85% to 93%.

Mitogen stimulation assay and mixed lymphocyte responses. Mitogenic stimulation responses of splenic lymphocytes were performed using the microtechnique of Strong et al.[17] Rat spleen cells were suspended in modified RPMI 1640 medium that consisted of equal parts of RPMI 1640 and EHAA medium.[18] This basic medium was supplemented with 17% serum (various proportions of fetal bovine serum and rat serum), insulin (5 μg/ml final concentration, Sigma, St. Louis, Missouri), and 2-mercaptoethanol (10^{-5} M final concentration). A nine-tenths-milliliter suspension of 1.1×10^6 spleen cells/ml was mixed with 0.1 ml of medium alone or medium containing various mitogens. Phytohemagglutinin (PHA, Wellcome, Greenville, North Carolina), lipopolysaccharide (LPS, Difco, Detroit, Michigan), pokeweed mitogen (PWM, Calbiochem, La Jolla, California), and concanavalin A (con A, Calbiochem) were used at final concentrations of 5 μg/ml, which produced maximum stimulation with spleen cells from both diabetic and normal rats (data not shown). Two-tenths-ml aliquots of this spleen cell-mitogen mixture were then distributed into 3–4 wells of a microtiter culture plate. The cultures were kept at 37°C in a 5% CO_2 humidified air atmosphere for 96 h. Sixteen hours before harvest, one μCi [methyl-^{3}H]-thymidine in 50 μl of medium (New England Nuclear, Boston, Massachusetts) was added to each well. At the end of the incubation, cells were harvested and collected on glass fiber filter paper using a Mash II Cell Harvestor (M.A. Bioproducts, Walkersville, Maryland). The cell-containing glass fiber discs were air dried and placed into vials containing 2.5 ml of Scinti-Verse I (Fisher Scientific Co.). ^{3}H-thymidine was measured on a Beckman LS 7000 (Beckman Instruments) and expressed as count per min (cpm).

The mixed lymphocyte responses (MLR) of spleen cells between diabetic Wistar rats and normal Sprague-Dawley rats were performed in microtiter culture plates.[19] The two-way MLR consisted of a 1:1 mixture of spleen cells from both strains (10^6 cells/ml, 0.2 ml/well). Control cultures consisted of spleen cells from either strain cultured alone. Increase of ^{3}H-thymidine incorporation (Δcpm) was calculated by subtracting the cpm obtained in the mixed culture by one-half of the sum of cpm observed in the two control cultures. The stimulation index (SI) was calculated by dividing the cpm obtained in the mixed culture by one-half of the sum of cpm observed in the two control cultures. In one-way MLR the stimulator was treated with mitomycin-C (50 μg/ml) at 37°C for 45 min to block proliferation of the stimulator cells. Cultures were incubated for 96 h and harvested as described above.

RESULTS

Characteristics of peripheral blood and spleen cells from diabetic rats. Spleens of diabetic rats were visibly smaller than the spleens of normal rats. To study the immunity of diabetic rats, PBL and spleen cell counts were quantitated. The results showed that both leukocytes per mm^3 blood and total lymphocytes per spleen were significantly lower (both $P < 0.02$) in diabetic than in normal rats (Table 1). Differential counts of PBL cells (Table 1) showed that the percentage of lymphocytes in the diabetic rat was significantly decreased ($P < 0.02$), while the percentage of neutrophils was significantly increased ($P < 0.02$). There was no significant difference in the percentages of monocytes or eosinophils between diabetic and normal rats. Rats killed 30 min after streptozotocin injection had spleen cell, leukocyte, and differential cell counts of PBL similar to noninjected controls (data not shown).

Mitogenic stimulation responses and mixed lymphocyte responses of diabetic rat spleen cells. Since there was a quantitative decrease of PBL and spleen cell counts in diabetic rats, it was pertinent to determine if lymphoid cells from diabetic rats retained normal function. The in vitro mitogenic stimulation assay (Table 2) showed that the response to PHA was similar between diabetic and normal spleen cells. However, the responses to LPS, PWM, and con A were consistently decreased in diabetic spleen cells when compared with normal spleen cells. Only the con A-stimulated group showed a statistically significant difference ($P < 0.025$). Spleen cells from rats killed 30 min after streptozotocin injection responded to mitogens similarly to cells from normal rats. Serum triglyceride values of these rats were within the normal ranges (data not shown).

Since con A is capable of binding to VLDL,[20] and since spleen cells isolated from diabetic rats may be exposed to elevated levels of VLDL, it was pertinent to determine if con A might be binding to VLDL on the surface of cells from diabetic rats, thus reducing its effective concentration. To

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TABLE 1
Characteristics of peripheral blood leukocytes and spleen cells from normal and diabetic rats

	Normal	Diabetic
Lymphocytes (× 10^{-6})/spleen (N)	260 ± 23 (4)	123 ± 25 (3)*
Peripheral blood leukocytes/mm³ (N)	9833 ± 1499 (4)	4959 ± 171 (4)*
% Lymphocyte (range)	78.3 ± 2.2 (70–82)	60.5 ± 4.7 (54–74)*
% Monocyte (range)	4.3 ± 1.0 (0–7)	2.8 ± 0.9 (0–4)
% Neutrophil (range)	16.3 ± 2.5 (10–24)	36.2 ± 5.4 (22–46)*
% Eosinophil (range)	1.2 ± 0.5 (0–3)	0.5 ± 0.5 (0–2)

Peripheral blood collected in tubes containing heparin was counted in a Coulter counter to obtain the total leukocyte counts. Differential counts were determined by the Wright's staining technique. N indicates number of normal and diabetic rats (7 days after i.v. injection of 100 mg/kg of streptozotocin). Data shown as mean ± SE.
* $P < 0.02$ compared with normal.

eliminate this possibility we measured the responses of spleen cells from diabetic and normal rats to three different concentrations of each of the mitogens. Tripling the concentrations of mitogens from 5 μg/ml to 15 μg/ml never increased the response of diabetic spleen cells to the level of normal spleen cells. In one experiment, the ³H-thymidine incorporations (cpm) of diabetic spleen cells were 16,737, 28,394, and 27,120 for con A doses of 5 μg/ml, 10 μg/ml, and 15 μg/ml, respectively, whereas the responses of normal spleen cells were 48,275, 36,215, and 38,375 for con A doses of 5 μg/ml, 10 μg/ml, and 15 μg/ml, respectively. The differences between the responses of diabetic and normal spleen cells at each con A concentration were statistically significant ($P < 0.01$). Although there is an increase in ³H-thymidine incorporation with the diabetic spleen cells from 5 to 10 μg of con A, this is not a consistent finding, and 5 μg/ml was judged to produce maximal stimulation.

TABLE 2
Mitogenic stimulation responses of spleen cells from normal and diabetic rats

Mitogens	Δ counts/min ± SE (× 10^{-3}) Normal	Diabetic
PHA	40.0 ± 4.9	39.0 ± 14.4
LPS	2.7 ± 1.6	0.7 ± 0.7
PWM	55.6 ± 13.2	28.0 ± 5.3
Con A	56.9 ± 9.2	23.2 ± 6.6*

Spleen cells (2 × 10⁵/well) in 0.2 ml of modified RPMI 1640 supplied with 13% fetal bovine serum and 4% normal rat serum were cultured alone or with phytohemagglutinin (PHA), lipopolysaccharide (LPS), pokeweed mitogen (PWM), or concanavalin A (con A) at 37°C in 5% CO_2 in air for 96 h. The final concentration of mitogens was 5 μg/ml. ³H-thymidine was added 18 h before harvesting. Cells were harvested and counted in a liquid scintillation counter. N = 4 for both the normal and diabetic groups. The background (cpm) without mitogens was 3587 and 2922 for normal and diabetic spleen cells, respectively.
* $P < 0.025$ when compared with normal.

We then attempted to determine if the decreased response was due to the induction of suppressor cells in the diabetic rats. To determine this, normal spleen cells were co-cultured with diabetic spleen cells, which were either treated or untreated with mitomycin-C (Table 3). In experiment 1, the response of the normal and diabetic spleen cell mixture is equal or slightly higher than one-half of the sum of the responses of normal and diabetic cells cultured alone. After mitomycin-C treatment (Table 3, experiment 2), the background proliferation of diabetic spleen cells alone was completely blocked (32 ± 1 cpm), thus leaving the observed response due solely to the normal spleen cells in the culture. The responses of the mixed normal and mitomycin-C-treated diabetic cell cultures were approximately one-half of the normal cultures, which had twice as many cells (Table 3, experiment 2). Thus, it was considered unlikely that suppressor cells are induced in diabetic rats.

Mixed lymphocyte responses (MLR) between diabetic Wistar rats and normal Sprague-Dawley rats demonstrated a good response in both two-way and one-way MLR

TABLE 3
Failure of diabetic rat spleen cells to suppress the mitogenic responses of normal spleen cells

Treatment of diabetic cells in mixed culture	Mitogens	counts/min ± SE Normal	½ normal ± ½ diabetic	Diabetic
None (experiment 1)	None	188 ± 26	1407 ± 141	3535 ± 370
	PHA	1133 ± 21	4225 ± 181	8765 ± 108
	LPS	94 ± 6	2394 ± 208	5206 ± 790
	PWM	2797 ± 50	13095 ± 887	17659 ± 1418
	Con A	8200 ± 387	8833 ± 815	7150 ± 638
Mitomycin-C (experiment 2)	None	1180 ± 185	874 ± 205	2096 ± 594
	PHA	35180 ± 5979	29138 ± 951	45281 ± 2970
	LPS	2209 ± 243	1244 ± 105	3990 ± 851
	PWM	36297 ± 1544	30262 ± 896	36476 ± 7159
	Con A	48389 ± 8542	27417 ± 5232	32852 ± 337

All cultures contained a total of 2 × 10⁵ spleen cells/well. Culture time was 96 h. The diabetic spleen cells in the mixed cultures were either untreated or treated with 50 μg/ml of mitomycin-C. CPM for mitomycin-C–treated diabetic cells used in the mixed cultures were 32 ± 1. The final concentrations of mitogens were 5 μg/ml.

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TABLE 4
Mixed lymphocyte responses of diabetic and normal rat spleen cells

Spleen cell culture	Experiment 1			Experiment 2		
	cpm ± SE	Δcpm	SI	cpm ± SE	Δcpm	SI
Diabetic	1618 ± 1	—	—	4438 ± 982	—	—
Normal	8878 ± 900	—	—	8878 ± 900	—	—
MC-treated normal	235 ± 97	—	—	235 ± 97	—	—
Diabetic + normal (two-way)	27191 ± 934	21943	5.2	33053 ± 2123	26395	5.0
Diabetic + MC-treated normal (one-way)	8431 ± 546	7504	9.1	17619 ± 479	15292	7.6

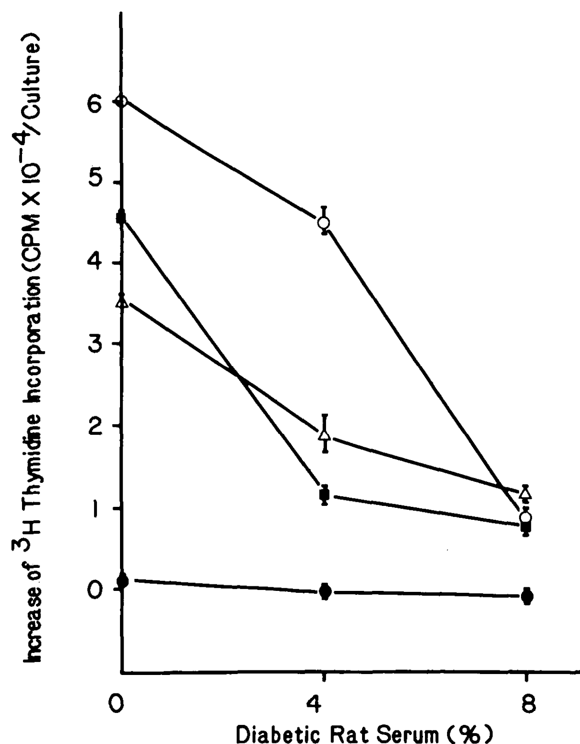
Spleen cells (2×10^5/well) were cultured in modified 1640 medium supplemented with 13% fetal bovine serum and 4% normal rat serum for 4 days. In the mixed cultures, 10^5 diabetic (Wistar) spleen cells were cultured with either 10^5 normal or 10^5 mitomycin-C–treated normal (Sprague-Dawley) spleen cells. Δcpm = cpm of mixed culture − ½(cpm of diabetic + cpm of normal).

$$(SI) = \frac{\text{cpm of mixed culture}}{½(\text{cpm of diabetic}) + ½(\text{cpm of normal})}$$

(Table 4). However, the stimulation index of the one-way MLR, using diabetic spleen cells as responders, was much higher than the two-way MLR, suggesting that diabetic spleen cells responded normally in MLR.

Toxic activity of diabetic serum and VLDL from diabetic serum on the mitogenic responses of normal rat spleen cells. Our previous study has shown that there is an elevated level of very-low-density lipoproteins (VLDL) in the diabetic serum that is toxic to porcine aortic endothelial cells in vitro.[12] To determine if a similar toxicity may be affecting spleen cells in the diabetic rats, diabetic serum was incubated with normal rat spleen cells and the mitogenic responses determined. Increasing percentages of diabetic serum in the culture medium decreased the mitogenic stimulation responses of normal rat spleen cells (Figure 1). The diabetic serum used in these experiments contained 1171 mg triglyceride/dl serum, which is approximately 10 times the normal nonfasting level of triglycerides.

FIGURE 1. Inhibition of mitogenic responses of rat spleen cells by diabetic serum. Modified RPMI 1640 medium was supplied with 13% fetal bovine serum and either 0%, 4%, or 8% diabetic serum. The ^{3}H-thymidine incorporation without mitogens was 8878, 11,665, and 3528 cpm for 0%, 4%, and 8% diabetic serum cultures, respectively. Mitogens used were PHA (■—■), PWM (○—○), con A (△—△), and LPS (●—●).



VLDL prepared from diabetic rat serum by ultracentrifugation were tested for their effect on the mitogenic stimulation responses of normal rat spleen cells. Inclusion of VLDL at 524 μg triglyceride/ml final concentration severely inhibited incorporation of ^{3}H-thymidine in both control cultures and cultures stimulated with LPS, PWM, con A, and PHA with values ranging from 8978 ± 900 cpm to 73,246 ± 4886 cpm without VLDL to values of 80 ± 29 cpm to 477 ± 286 cpm with VLDL. Since VLDL at 524 μg triglyceride/ml was totally toxic to rat spleen cells, the effects of various concentrations of VLDL on ^{3}H-thymidine incorporation, total cell counts, and cell viability in unstimulated cultures and cultures stimulated with PHA were measured. Addition of increasing amounts of VLDL to spleen cell cultures resulted in an exponential decrease in ^{3}H-thymidine incorporation. The regression coefficient for unstimulated spleen cells was −0.00272 ($P < 0.01$) and for PHA-stimulated spleen cells was −0.00023 ($P < 0.001$); the correlation coefficients were 0.836 and 0.991, respectively. At a final concentration of 65 μg triglyceride/ml, VLDL inhibited the proliferation response induced by PHA to more than one-half of the control value and complete inhibition was achieved at a concentration of 262 μg triglyceride/ml (Figure 2). Examination of the lymphocyte cultures at the end of the incubation revealed that increasing VLDL decreased viability of culture cells. The viabilities of the cells in both 262 and 524 μg VLDL triglyceride/ml were only 11–12% compared with 58% in cultures without added VLDL ($P < 0.005$) (Table 5). The absolute cell numbers per culture also decreased with increasing concentrations of VLDL (Table 5). These results indicated that VLDL isolated from diabetic rat serum is toxic to rat lymphocytes.

To further substantiate the toxicity of elevated levels of VLDL, VLDL were removed from diabetic serum by ultracentrifugation. The infranatant ($d > 1.006$) from diabetic rat serum supported growth and allowed the cells to remain as viable as cells cultured in normal rat serum (Tables 5 and 6). The viabilities of 4-day-old spleen cells in infranatant diabetic serum were 52% (PHA-stimulated) and 61% (control), which were similar to those of cells in normal rat

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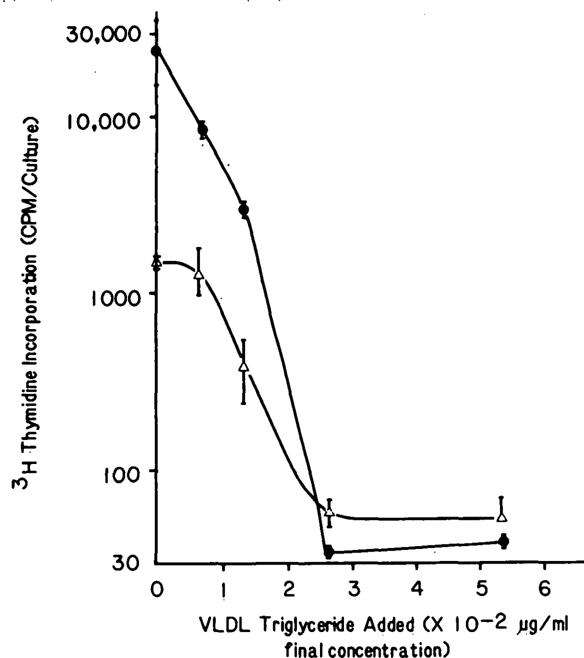

FIGURE 2. Inhibition of PHA responses of normal rat spleen cells by very-low-density lipoprotein (VLDL) from diabetic rat serum. Modified RPMI 1640 medium was supplied with 13% fetal bovine serum and 4% normal rat serum. The culture time was 96 h either with (●—●) or without (△—△) PHA.

serum (Tables 5 and 6). When VLDL were added back to the diabetic serum infranatant, toxicity was again restored (Table 6). The differences in viability, cell number per culture, and ^{3}H-thymidine incorporation between infranatant diabetic serum (d > 1.006) and intact diabetic serum or infranatant diabetic serum reconstituted with VLDL were all statistically significant ($P < 0.01$) (Table 6).

DISCUSSION

Evidence of immune impairment in poorly controlled diabetic individuals has been reported from a number of sources and includes a decrease in antibody production,[21,22] alterations in certain subpopulations of T-cells,[23,24] a decrease in delayed type hypersensitivity,[21,25] a decrease in the production of migration inhibition factor,[26] a decrease in the mitogenic stimulation responses,[27] and an increase in the survival time of allografts.[25] Although the nature of the suppression of immunity in diabetes is not clear, several reports studying streptozotocin-induced diabetic mice have suggested that this phenomenon may be related to decreases in weight and numbers of nucleated cells in lymphoid organs.[21,28–30] Our results showing a significant decrease in the absolute number of spleen lymphocytes and PBL in streptozotocin-induced diabetic rats agree with these reports. In addition, there is also a differential shift in PBL in diabetic rats as evidenced by a decreasing percentage of lymphocytes and increasing percentage of neutrophils. This may indicate that lymphocytes are more prone to injury in the diabetic state than neutrophils.

B- and T-cell mitogens and mixed culture responses were used to determine the quality of immunocompetent cells in diabetic rats. While MLR and PHA responses were normal, the responses to mitogens (PWM, LPS, and con A) were consistently decreased. Because of the inherent variation from experiment to experiment only the con A-stimulated cells demonstrated a statistically significant decrease. The large decreases in the mitogenic responses of diabetic rat spleen cells were not due to the toxicity of streptozotocin itself, since its half-life in blood is 15 min,[31–33] and responses of spleen cells from rats injected with streptozotocin 30 min before death were found to be normal. Increasing concentrations of con A did not increase the stimulation responses of diabetic spleen cells, indicating that the cells were maximally stimulated at the concentrations of con A used. Thus, the decreased response of diabetic spleen cell cultures to mitogens was not due to a reduced availability of con A molecules, which may have been bound by possibly increased amounts of VLDL in the diabetic spleen cell preparations. Therefore, the substantial decrease in mitogenic responses of spleen cells from uncontrolled diabetic rats is probably due to the poor proliferation of impaired lymphocytes in the diabetic state.

Recently we have reported that diabetic rat serum is toxic to porcine aortic endothelial cells in culture and have shown that the toxic activity is located in VLDL.[12] We thus decided to determine the effects of diabetic rat serum and VLDL on the mitogenic responses of normal rat spleen cells. Addition of either diabetic serum or VLDL inhibited the incorporation of ^{3}H-thymidine (Figures 1 and 2). This inhibition is not due

TABLE 5
Toxicity of VLDL from diabetic rat serum on rat spleen cell cultures

VLDL triglyceride added (µg/ml final concentration)	Control		+PHA	
	% Viability	Cells/culture (× 10⁻⁴)	% Viability	Cells/culture (× 10⁻⁴)
0	58.0 ± 6.8	4.2 ± 0.5	64.5 ± 3.8	24.4 ± 1.2
65	48.7 ± 2.2	4.8 ± 0.5	62.7 ± 0.3	10.4 ± 0.7§
131	21.0 ± 4.9‡	3.0 ± 1.0	50.0 ± 2.9*	5.4 ± 1.2§
262	11.3 ± 1.8†	1.6 ± 0.3‡	39.3 ± 3.7‡	4.2 ± 0.7§
524	12.6 ± 0.3†	2.2 ± 0.2‡	31.3 ± 5.0‖	3.6 ± 0.5§

Spleen cells (2 × 10^5/culture) in 0.2 ml of modified RPMI 1640 supplied with 13% fetal bovine serum and 4% normal rat serum and various concentrations of very-low-density lipoprotein (VLDL) were cultured alone or with 5 µg/ml PHA at 37°C in 5% CO_2 in air for 96 h. Cultures were performed in triplicate. Viability of spleen cells was 92% at culture time.
* $P < 0.05$ compared with non-VLDL culture.
† $P < 0.005$ compared with non-VLDL culture.
‡ $P < 0.025$ compared with non-VLDL culture.
§ $P < 0.001$ compared with non-VLDL culture.
‖ $P < 0.01$ compared with non-VLDL culture.

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TABLE 6
Association of diabetic rat serum toxicity with the very-low-density lipoprotein fraction

		Control culture			PHA-stimulated culture		
Serum added	Triglycerides (mg/dl serum)	% Viability	Cells/culture ($\times 10^{-4}$)	^{3}H-thymidine incorporation (cpm)	% Viability	Cells/culture ($\times 10^{-4}$)	^{3}H-thymidine incorporation (cpm)
DRS	438	11.0 ± 2.7†	2.1 ± 0.6†	36 ± 1‡	9.3 ± 1.8†	0.9 ± 0.2*	36 ± 1†
DRS-VLDL-depleted	168	60.5 ± 2.3	10.0 ± 0.3	1503 ± 313	51.8 ± 1.9	15.5 ± 0.6	25251 ± 2154
DRS-VLDL-depleted + VLDL (0.03 ×)	177	36.8 ± 4.2*	6.4 ± 0.6*	707 ± 51‡	32.3 ± 1.5†	5.8 ± 0.2*	9754 ± 219†
DRS-VLDL-depleted + VLDL (0.1 ×)	193	19.8 ± 1.9†	3.0 ± 0.6†	268 ± 49*	14.8 ± 2.2†	2.1 ± 0.5*	5263 ± 98†
DRS-VLDL-depleted + VLDL (1.0 ×)	422	8.5 ± 8.7*	3.4 ± 1.6*	40 ± 2*	8.8 ± 3.4†	1.1 ± 0.3*	34 ± 5†

Spleen cells (2×10^5/culture) in 0.2 ml of modified 1640 were supplemented with either diabetic rat serum (DRS), the d > 1.006-ultracentrifugation fraction of DRS (DRS-VLDL-depleted), or the DRS-VLDL-depleted fraction to which various concentrations of VLDL had been added back, and cultured alone or with 5 μg/ml of PHA at 37°C in 5% CO_2 in air for 96 h. Cultures were performed in quadruplicate. Viability of spleen cells was 97% at culture time. Data expressed as mean ± SE.
* $P < 0.01$ compared with DRS-VLDL-depleted culture.
† $P < 0.001$ compared with DRS-VLDL-depleted culture.
‡ $P < 0.05$ compared with DRS-VLDL-depleted culture.

to complement, since heat-inactivated sera retain this inhibitory effect (data not shown). We have reported that in vivo insulin treatment of diabetic rats reversed the toxicity of diabetic rat serum to porcine aortic endothelial cells in culture, while addition of insulin in vitro did not correct the toxicity of diabetic rat serum.[12] The toxicity is correlated with serum triglyceride levels but not glucose or cholesterol.[12] Since the medium used in the present study was supplied with 5 μg/ml insulin, the inhibitory effect of diabetic serum is not due to the lack of insulin in the spleen cell cultures. Our previous study shows that addition of VLDL to porcine aortic endothelial cell cultures causes cellular contraction and the cells to become permeable to trypan blue, resulting in cell death and finally detachment. This toxicity of VLDL was not due to the peroxidation of lipid during the preparation and incubation of VLDL.[12] We have also reported that elevated levels of VLDL isolated from either normal or diabetic rat serum demonstrate the same degree of toxicity when added to porcine aortic endothelial cells at equivalent triglyceride concentrations.[34] Addition of VLDL (262 μg triglyceride/ml) significantly ($P < 0.005$) decreased the viability of 4-day-old lymphocyte cultures (Table 5), indicating that the inhibitory effect is due to the toxicity of VLDL. These results extend the observation of VLDL toxicity in a heterologous system[12] to toxicity in a homologous system using both lymphocytes and serum from the same species.

Curtiss and Edgington[35] have reported that human serum low-density lipoprotein (LDL) possesses inhibitory activity for mitogenic responses and MLR. This inhibitory activity of LDL is noncytotoxic. Hui et al.[36] recently have also reported that LDL as well as high-density lipoproteins suppress PHA-induced lymphocyte activation and that the increase of effectiveness in suppression is due to the binding of receptors on lymphocytes. However, from our results the impaired immunologic function in severe diabetes appears to be due to injury of essentially normal lymphocytes by elevated levels of VLDL. Purification and identification of the biologically active component is currently under investigation in order to further characterize the nature of the VLDL toxicity on lymphocytes and aortic endothelial cells.

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REFERENCES

1 Huvos, A., and Rocha, H.: Frequency of bacteremia in patients with diabetes mellitus. N. Engl. J. Med. *261*:1213–16, 1959.
2 Kass, E.: Hormones and host resistance to infection. Bacterial Rev. *24*:177–85, 1960.
3 Pmetta, D., Rees, S. B., Younger, D., and Kass, E. H.: A symptomatic bacteriuria in diabetes mellitus. N. Engl. J. Med. *276*:1118–21, 1967.
4 Savin, A. J.: Bacterial infections in diabetes mellitus. Br. J. Dermatol. *91*:481–87, 1974.
5 Bagdade, J. D.: Infection. *In* Diabetes Mellitus: Diagnosis and Treatment. Vol. 3. Fajans, S. S., and Sussman, K. E., Eds. New York, American Diabetes Association, 1971, pp. 214–15.
6 Perillie, P. E., Nolan, J. P., and Finch, S. C.: Studies of the resistance to infection in diabetes mellitus: local exudative cellular response. J. Lab. Clin. Med. *59*:1008–1015, 1962.
7 Bybee, J. D., and Rogers, D. E.: The phagocytic activity of polymorphonuclear leukocytes obtained from patients with diabetes mellitus. J. Lab. Clin. Med. *64*:1–13, 1964.
8 Brody, J. I., and Mertie, K.: Metabolic and biosynthetic features of lymphocytes in chronic lymphatic leukemia. Br. J. Hematol. *19*:193–201, 1971.
9 Mowat, A. G., and Baum, J.: Chemotaxis of polymorphonuclear leukocytes from patients with diabetes mellitus. N. Engl. J. Med. *284*:621–27, 1971.
10 Bagdade, J. D., Root, R. K., and Bulger, R. J.: Impaired leukocyte functions in patients with poorly controlled diabetes. Diabetes *23*:9–15, 1974.
11 Plouffe, J. F., Silva, J., Jr., Fekety, R., and Allen, J. L.: Cell-mediated immunity in diabetes mellitus. Infect. Immun. *21*:425–29, 1978.
12 Arbogast, B. W., Lee, G. M., and Raymond, T. L.: In vitro injury of porcine aortic endothelial cells by very low density lipoproteins from diabetic rat serum. Diabetes *31*:593–99, 1982.
13 Junod, A., Lambert, A. E., Ore, L., Pictet, R., Gonet, A. E., and Renold, A. E.: Studies of the diabetogenic action of streptozotocin. Proc. Soc. Exp. Biol. Med. *126*:210–15, 1967.
14 Koga, S., Horowitz, D. L., and Scanu, A. M.: Isolation and properties of lipoproteins from normal rat serum. J. Lipid Res. *10*:577–88, 1969.
15 Noble, R. P.: Electrophoretic separation of plasma lipoproteins in agarose gel. J. Lipid Res. *9*:693–700, 1968.
16 Bucolo, G., and Davis, H.: Quantitative determination of serum triglycerides by use of enzymes. Clin. Chem. *19*:476–82, 1973.
17 Strong, D. M., Ahmed, A. A., Thurman, G. B., and Sell, K. W.: In vitro stimulation of murine spleen cells using a microculture system and a multiple automated sample harvester. J. Immunol. Methods *2*:279–91, 1973.
18 Click, R. E., Benck, L., and Alter, B. J.: Immune responses in vitro. 1. Culture conditions for antibodies synthesis. Cell. Immunol. *3*:264–76, 1972.

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[19] Hodes, R. J., and Terry, W. D.: Comparison of irradiated and mitomycin-C-treated mouse spleen cells as stimulating cells in mixed lymphocyte cultures and in vitro sensitization. J. Immunol. *113*:39–44, 1974.

[20] Rouffy, J., Bakir, R., Chanu, B., Goy-Loeper, J., Abou, A., Walti, H., Goussault, Y., Neel, D., Dakkak, R., and Dreux, C.: Comparative study of total cholesterol, HDL cholesterol, LDL + VLDL cholesterol. Two methods: precipitation by concanavalin A and ultra centrifugation. Ann. Biol. Clin. (Paris) *38*:305–308, 1980.

[21] Ishibashi, I., Kitahara, Y., Harada, Y., Harada, S., Takamoto, M., and Ishibashi, T.: Immunologic features of mice with streptozotocin-induced diabetes. Depression of their immune responses to sheep red blood cells. Diabetes *29*:516–23, 1980.

[22] Ludwig, H., Eibl, M., Schernthaner, G., Erd, W., and Mayr, W. R.: Humoral immunodeficiency to bacterial antigens in patients with juvenile onset diabetes mellitus. Diabetologia *12*:259–62, 1976.

[23] Pozzilli, P., DiMario, U., Javicoli, M., Menzinger, G., and Andreani, D.: Inhibition of allogeneic lymphocyte E-rosettes induced by sera from newly diagnosed type I diabetics. Acta Endocrinol. *93*:49–53, 1980.

[24] Miller, R., Kolb, H., Kuschak, D., Jorgens, V., and Gries, F.A.: Analysis of T-lymphocyte subpopulations in juvenile-onset diabetics. Clin. Exp. Immunol. *39*:130–35, 1980.

[25] Mahmoud, A. A. F., Rodman, H. M., Mandel, M. A., and Warren, K. S.: Induced and spontaneous diabetes mellitus and suppression of cell-mediated immunologic responses. Granuloma formation, delayed dermal reactivity and allograft rejection. J. Clin. Invest. *57*:362–67, 1976.

[26] Kolterman, O. G., Olefsky, J. M., Kurahara, C., and Taylor, K.: A defect in cell-mediated immune function in insulin-resistant diabetic and obese subjects. J. Lab. Clin. Med. *96*:535–43, 1980.

[27] Eliashiv, A., Olumide, F., Norton, L., and Eiseman, B.: Depression of cell-mediated immunity in diabetes. Arch. Surg. *113*:1180–83, 1978.

[28] Nichols, W. K., Spellman, J. B., and Daynes, R. A.: Immune responses of diabetic animals. Comparison of genetically obese and streptozotocin-diabetic mice. Diabetologia *14*:343–44, 1978.

[29] Pavelic, K., Slijepcevic, M., and Pavelic, J.: Recovery of immune system in diabetic mice after treatment with insulin. Horm. Metab. Res. *10*:381–86, 1978.

[30] Nichols, W. K., Spellman, J. B., Vann, L. L., and Daynes, R. A.: Immune responses of diabetic animals. Direct immunosuppressant effects of streptozotocin in mice. Diabetologia *16*:51–57, 1979.

[31] Schein, P. S., Kahn, R., Gorden, P., Wells, S., and DeVita, V. T.: Streptozotocin for malignant insulinomas and carcinoid tumor. Arch. Intern. Med. *132*:555–61, 1973.

[32] Saiki, O., Negoro, S., Tsuyuguchi, I., and Yamamura, Y.: Depressed immunological defense mechanisms in mice with experimentally induced diabetes. Infect. Immun. *28*:127–31, 1980.

[33] Ptak, W., Rewicka, M., and Kollar, M.: Development of specific suppressor cells in hypoinsulinaemic mice. Nature *283*:199–200, 1980.

[34] Arbogast, B. W., Lee, G. M., and Raymond, T. L.: In vitro injury of porcine aortic endothelial cells by very low density lipoproteins from diabetic and normal rat serum. Fed. Proc. *41*:454, 1982.

[35] Curtiss, L. K., and Edgington, T. S.: Regulatory serum lipoproteins: regulation of lymphocytes stimulation by a species of low density lipoprotein. J. Immunol.*116*:1452–58, 1976.

[36] Hui, D. Y., Harmony, J. A. K., Innerarity, T. L., and Mahley, R. W.: Immunoregulatory plasma lipoproteins. Role of apoprotein E and apoprotein B. J. Biol. Chem. *24*:11,775–81, 1980.

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