

Altered Cytokine and Nitric Oxide Secretion In Vitro by Macrophages From Diabetic Type II-Like *db/db* Mice

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Macrophage dysfunction is a likely mechanism underlying common diabetic complications such as increased susceptibility to infection, accelerated atherosclerosis, and disturbed wound healing. There are no available studies on the function of tissue macrophages in diabetes in humans. We have therefore studied peritoneal macrophages from diabetic type 2-like *db/db* mice. We found that the release of tumor necrosis factor- α and interleukin-1 β from lipopolysaccharide plus interferon- γ -stimulated macrophages and vascular endothelial growth factor from both stimulated and nonstimulated macrophages was significantly reduced in diabetic animals compared with nondiabetic controls. Nitric oxide production from the stimulated *db/db* macrophages was significantly higher than that in the *db/+* cultures, whereas there was no difference in their ability to generate reactive oxygen species. When studied both at light and electron microscopic levels, macrophages in diabetic animals had an altered morphological appearance compared with those of normal controls. We conclude that the function and morphology of the macrophages are disturbed in *db/db* mice and that this disturbance is related to the mechanisms underlying common inflammatory and degenerative manifestations in diabetes. *Diabetes* 49:1451–1458, 2000

Several common diabetic complications, such as increased susceptibility to infection, accelerated atherosclerosis, and disturbed wound healing, can be explained by defects in nonspecific immunity, inflammation, and tissue repair. This explanation makes the macrophage system an obvious target in studies of the mechanisms underlying these complications.

Previous studies of the function of the macrophage system in human diabetes have been performed on blood monocytes. The data from these studies are difficult to interpret with respect to the in vivo situation because the functional mono-

nuclear phagocytes in situ are tissue macrophages, which are a distinct subset of cells with a phenotype different from that of free-floating monocytes. Data on tissue macrophages from patients with diabetes are not available, which motivates careful studies of relevant animal models. Studies on DP-BB rats, streptozotocin-treated rats, and alloxan-treated diabetic mice—all type 1-like—have revealed altered (mostly decreased) release of cytokines and growth factors from macrophages in vitro (1–3). Only a few investigations have been performed on macrophages from animals with type 2-like conditions, e.g., *db/db* mice. Tissue macrophages from these animals display reduced phagocytosis and intracellular killing of microbes (4) as well as changed morphology during experimental inflammation in vivo (5). The present study was undertaken to determine whether the secretion of cytokines, growth factors, and other substances highly relevant to nonspecific immunity, inflammation, and repair were disturbed in macrophages from *db/db* mice. We report the decreased in vitro release of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , and vascular endothelial growth factor (VEGF) as well as increased release of nitric oxide (NO) from tissue macrophages of *db/db* mice. We also demonstrate morphological abnormalities in these cells, probably related to a compromised function in vivo.

RESEARCH DESIGN AND METHODS

Animals. Male diabetic C57BL/KS-lepr-*db/db* and nondiabetic C57BL/KS-lepr-*db/+* mice purchased from Harlan (Blackthorn, U.K.) were kept under controlled animal room conditions at $21 \pm 1^\circ\text{C}$, relative humidity $55 \pm 10\%$, with a 12:12 light:darkness cycle (0800–2000). The animals were fed standard maintenance rodent diet (B&K, Nittedal, Norway) and received water ad libitum. The experimental protocols were approved by the Norwegian Ethics Committee for Research on Animals.

Experimental procedure. All the experiments started between 1200 and 1400. After 4 h of fasting, blood for measurements of glucose, lactate, HbA_{1c}, and insulin was obtained from orbital vessels under terminal anesthesia with the mixture of Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) and Dormicum (F. Hoffmann-La Roche, Basel) (final concentrations: 0.05 mg/ml Fentanyl, 2.5 mg/ml Fluanison, 1.25 mg/ml Midazolam; dose: 0.0075 mg/kg body wt).

When blood sampling was not needed, animals were killed by CO₂ suffocation, and resident peritoneal cells were harvested by lavage with 5 ml ice-cold modified RPMI 1640 with synthetic serum replacement medium (SSR-II; Mediatec, Copenhagen, Denmark) (RPMI 1640-SSR-II with 10 mmol/l glucose mixed in equal volumes with Earle's balanced salt solution made without glucose) with a final glucose concentration of 5 mmol/l. The yield of peritoneal cells from the diabetic animals was not significantly different from nondiabetic mice when calculated per gram of body weight ($206,165 \pm 9,104$ vs. $219,648 \pm 22,872$; $P > 0.05$). Cells were seeded at a density of 2×10^5 cells per 200 μl per well into Falcon 96-well plates (Becton Dickinson, Franklin Lakes, NJ; catalog number 353072) and cultured at 37°C in a humidified atmosphere at 5% CO₂. After 2 h, nonadherent cells were washed off, and the adherent cells (~98% macrophages, as judged by May-Grunwald-Giemsa staining) were cultured in the same modified SSR-II medium overnight. Fresh medium was added the next

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AGE, advanced glycation end product; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; NO₂⁻, nitrite; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

morning, and cells were incubated with 10 µg/ml phorbol myristate acetate (PMA) (Sigma, St. Louis, MO), 1 µg/ml lipopolysaccharide (LPS) (*Escherichia coli*: O26:B6 LPS; Difco Laboratories, Detroit, MI), 100 U/ml interferon (IFN)-γ (Genzyme, Cambridge, MA), or both LPS plus IFN-γ (the same concentrations). Supernatants were collected at 12, 24, and 48 h after stimulation. Unstimulated cultures served as controls.

Metabolic parameters. Glucose and lactate levels in serum of the animals were measured with the YSI Glucose and L-Lactate Analyzer Model 23005 STAT (Yellow Springs Instruments, Yellow Springs, OH), HbA_{1c} was assayed with a DCA 2000 Analyzer (Bayer Corporation, Elkhart, IN), and serum insulin levels were measured with a rat insulin radioimmunoassay kit (Linco Research, St. Charles, MO).

TNF-α, IL-1β, and VEGF assays. TNF-α, IL-1β, and VEGF concentrations in cell culture supernatants were measured with commercial enzyme-linked immunosorbent assay kits purchased from Biosource Europe, S.A. (Nivelles, Belgium) (TNF-α and IL-1β kits) and R&D Systems Europe (Abingdon, U.K.) (VEGF kit).

Nitric oxide release. Nitrite (NO₂⁻), which is a stable end product of NO, was measured by a colorimetric assay based on the Griess reaction (6). Briefly, 50 µl culture supernatants were incubated for 10 min at room temperature with 50 µl Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% H₃PO₄). The absorbance at 590 nm was determined using a microplate reader. The concentration of NO₂⁻ was calculated using a standard curve established with sodium nitrite.

Respiratory burst. The respiratory burst was measured in *db/db* and *db/+* macrophage cultures by estimating the O₂⁻-dependent reduction of the redox dye nitroblue tetrazolium to formazan (7).

DNA assay. The amount of DNA in the cultures was estimated after cell lysis in 100 µl of deionized water by measuring DABA-DNA fluorescence (DABA is from Sigma) at 420/510 nm (8).

Staining with crystal violet. Cells cultured in 24-well plates for 40 h were fixed in 1% glutaraldehyde (Sigma) in Hanks' balanced salt solution for 15 min, stained for 30 min in 0.1% crystal violet solution in deionized water, washed in water, and allowed to air-dry.

Scanning electron microscopy. Macrophages from both *db/db* and *db/+* mice were incubated in 24-well plates for either 2 or 24 h in medium containing 1 µg/ml LPS. Cultures incubated in medium alone served as nonstimulated controls. Cells were fixed in McDowell's fixative overnight, washed twice with phosphate-buffered saline (PBS), postfixed in 1% osmium tetroxide for 30 min, dehydrated in graded ethanols, and dried twice for 2 min in hexamethyldisilazane. The specimens were mounted on aluminum stubs, coated with gold, and examined in a JEOL 5310 scanning electron microscope (Tokyo).

Transmission electron microscopy. Immediately after lavage, peritoneal cells from both diabetic and control animals were fixed in McDowell's fixative overnight, washed twice in PBS, postfixed in 1% osmium tetroxide for 1.5 h, washed twice in PBS, rinsed twice in deionized water, stained for 1.5 h in 2% uranyl acetate in water, dehydrated in graded ethanols and propylene oxide, and embedded in Glycid Ether/Araldite (Serva, Heidelberg, Germany) according to standard procedures. After overnight polymerization at 60°C, samples were sectioned on a Reichert Ultracut S Ultramicrotome (Vienna), mounted on formvar-coated copper grids, and contrasted in 5% uranyl acetate and Reynolds lead citrate. The sections were examined in a JEOL (JEM 1010) transmission electron microscope.

Statistical analysis. The results are presented as means ± SE. Student's 2-tailed paired *t* test was applied for significance testing after checking for normality of distribution. All the statistical calculations were performed utilizing SAS 6.12 software package (SAS Institute, Cary, NC). *P* < 0.05 was considered statistically significant.

RESULTS

Metabolic characteristics of experimental animals. At the age of 2–3 months, C57BL/KS-*db/db* animals exhibited gross appearance and metabolic characteristics relevant to type 2 diabetes. They were obese and had elevated levels of blood glucose, lactate, HbA_{1c}, and serum insulin compared with heterozygous phenotypically normal animals of the same strain (Table 1).

DNA amounts (cell numbers). There was no significant difference in the DNA amounts (i.e., cell numbers) between diabetic and nondiabetic cultures at identical time points.

In vitro TNF-α and IL-1β release. The concentrations of TNF-α (Fig. 1) and IL-1β (Fig. 2A) were significantly lower in

TABLE 1
General characteristics of experimental animals

	<i>db/db</i>	<i>db/+</i>
Age	2–3 months	2–3 months
Body weight (g)	47.8 ± 1.8	20.1 ± 0.5
Serum glucose (mmol/l)	25.1 ± 4.9	8.6 ± 0.2
HbA _{1c} (%)	10.2 ± 0.9	4.1 ± 0.1
Serum insulin (pmol/l)*	2,446 ± 449	176 ± 64
Serum lactate	2.6 ± 0.3	1.7 ± 0.2

Data are means ± SE, unless otherwise indicated. *n* = 6; **n* = 5. Blood samples were obtained after 4 h of fasting.

cultures of peritoneal macrophages from diabetic mice compared with the phenotypically normal controls when stimulated with LPS plus IFN-γ. This result occurred at all the time points tested (e.g., TNF-α was 413 ± 57 vs. 772 ± 70 pg/ml at 12 h [*P* < 0.01], and IL-1β was 46 ± 11 vs. 166 ± 20 pg/ml at 48 h [*P* < 0.01]). Also, there was significantly less IL-1β in *db/db* cultures compared with *db/+* cultures when PMA or LPS alone was used as a stimulant (Fig. 2B). There was no significant difference in basic unstimulated TNF-α and IL-1β releases in the 2 categories of mice.

In vitro VEGF release. There was a 20% lower VEGF release from *db/db* macrophages stimulated with LPS plus IFN-γ for 12 h compared with *db/+* cells (3.3 ± 0.3 vs. 4.3 ± 0.3 pg/ml, *P* < 0.05) (Fig. 3). After 48 h of stimulation, there was an ~13- to 15-fold increase in VEGF levels in both groups. However, the level of VEGF was still significantly lower in the diabetic cultures than in the nondiabetic cultures (43.9 ± 7.9 vs. 65.0 ± 6.5 pg/ml, *P* < 0.01). There was no difference in VEGF levels between nonstimulated *db/db* macrophages incubated for 12 and 48 h (3.1 ± 0.1 vs. 1.9 ± 1.9 pg/ml). In nonstimulated *db/+* cells, however, the basal level of VEGF in the medium was increased ~3 times after 48 h (4.1 ± 0.5 vs. 11.4 ± 2.3 pg/ml, *P* < 0.05) and became significantly higher than that in the diabetic counterparts after 48 h of incubation.

In vitro NO release. We investigated the NO production in the cell culture supernatants 12 h after treatment of macro-

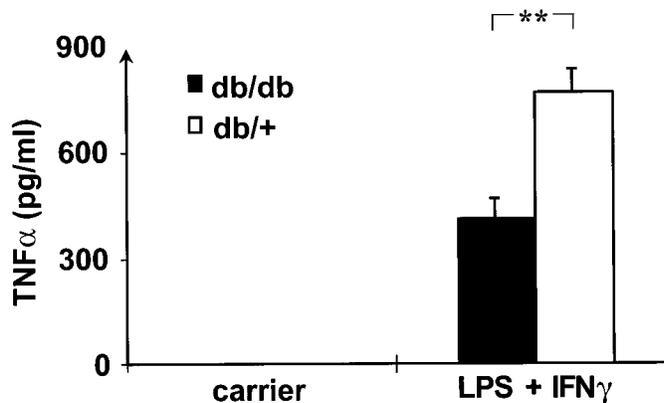


FIG. 1. In vitro TNF-α release from peritoneal macrophages in *db/db* and *db/+* mice. TNF-α was measured in the cell culture supernatants 12 h after LPS plus IFN-γ stimulation (1 µg/ml and 100 U/ml, respectively). Carrier: supernatants of nonstimulated cultures. Data are expressed as means ± SE; *n* = 9. ***P* < 0.01.

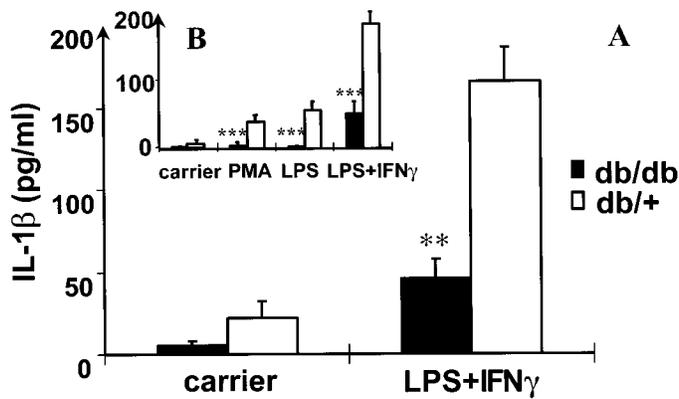


FIG. 2. In vitro IL-1 β release from peritoneal macrophages in *db/db* and *db/+* mice. **A:** IL-1 β was measured in the cell culture supernatants 48 h after LPS plus IFN- γ stimulation (1 μ g/ml and 100 U/ml, respectively). Data are expressed as means \pm SE; $n = 9$. ** $P < 0.01$ vs. nondiabetic controls. **B:** IL-1 β was measured in the cell culture supernatants 24 h after stimulation with PMA (10 μ g/ml), LPS (1 μ g/ml), or LPS plus IFN- γ (1 μ g/ml and 100 U/ml, respectively). Carrier: supernatants of nonstimulated cultures. Data are expressed as means \pm SE; $n = 6$. *** $P < 0.001$ vs. nondiabetic controls.

phages with LPS, IFN- γ , or both. The levels of NO $_2^-$ in culture supernatants were significantly higher in diabetic macrophages when stimulated with LPS (data not shown) or LPS plus IFN- γ compared with that of control cells (2.7 ± 0.4 μ mol/l in *db/db* vs. 0.8 ± 0.2 μ mol/l in *db/+*; $P < 0.01$) (Fig. 4). No NO $_2^-$ was detected in either diabetic or control cells in the unstimulated state or when stimulated with IFN- γ alone (data not shown).

Respiratory burst. No statistically significant difference was observed in PMA-induced respiratory burst between diabetic and nondiabetic macrophages (Fig. 5), as determined by intracellular O $_2^-$ accumulation.

Morphological appearance of macrophages. Diabetic macrophages cultured for 40 h—LPS-stimulated or not—

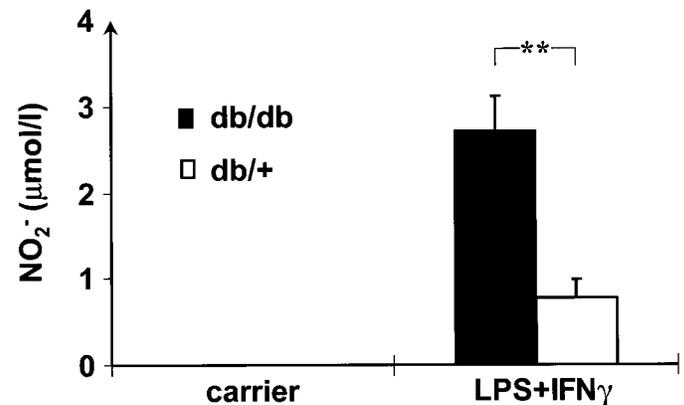


FIG. 4. In vitro NO release from peritoneal macrophages in *db/db* and *db/+* mice. NO $_2^-$ was measured in the cell culture supernatants 12 h after LPS plus IFN- γ stimulation (1 μ g/ml and 100 U/ml, respectively). Carrier: supernatants of nonstimulated cultures. Data are expressed as means \pm SE; $n = 9$. ** $P < 0.01$.

tended to be more rounded and clustered compared with the normal counterparts (Fig. 6). In scanning electron micrographs at early hours of culture, the number of spread cells appeared much higher among *db/+* macrophages (Fig. 7B) compared with *db/db* cells (Fig. 7A). Also, nondiabetic macrophages incubated for 24 h were typically covered with a network of thin filaments (possibly cytoplasmic extensions) (Fig. 7D) not seen in the diabetic cells (Fig. 7C and E). The filaments were more conspicuous in LPS-stimulated *db/+* cells (Fig. 7F).

As observed in transmission electron micrographs of cells in suspension, extensive peripheral vacuolization was a typical feature of diabetic peritoneal macrophages along with a certain rounded cell contour (Fig. 8A), whereas macrophages from control animals had fewer vacuoles of a smaller size and numerous cytoplasmic extensions (Fig. 8B). This difference in morphology was also evident after short-term culture (data not shown).

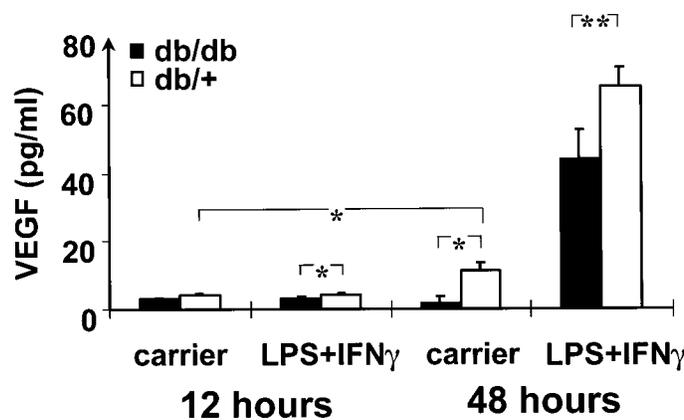


FIG. 3. In vitro VEGF release from peritoneal macrophages in *db/db* and *db/+* mice. VEGF was measured in the cell culture supernatants 12 and 48 h after LPS plus IFN- γ stimulation (1 μ g/ml and 100 U/ml, respectively). Carrier: supernatants of nonstimulated cultures. Data are expressed as means \pm SE; $n = 9$. * $P < 0.05$, ** $P < 0.01$.

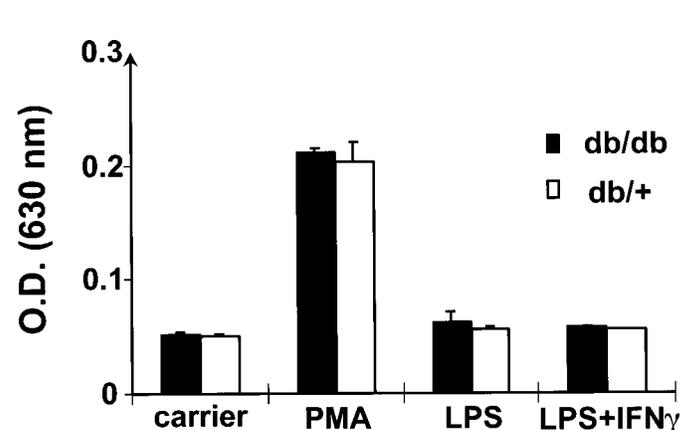


FIG. 5. Respiratory burst in peritoneal macrophages from *db/db* and *db/+* mice. Intracellular oxygen was measured by nitroblue tetrazolium reduction 24 h after stimulation with PMA (10 μ g/ml), LPS (1 μ g/ml), or LPS plus IFN- γ (1 μ g/ml and 100 U/ml, respectively). Carrier: supernatants of nonstimulated cultures. Data are expressed as means \pm SE; $n = 6$. $P > 0.05$ vs. nondiabetic controls. O.D., optical density.

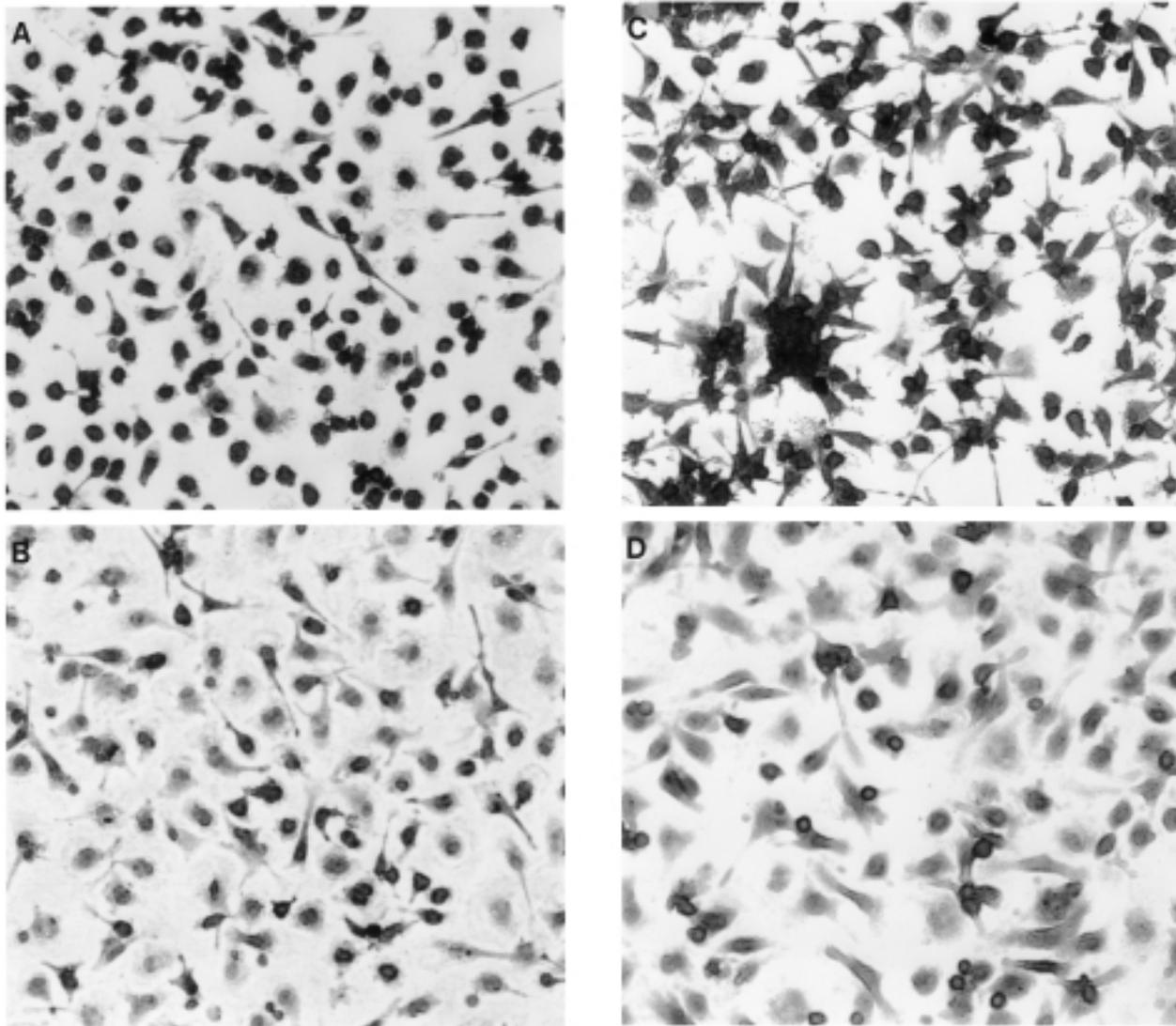


FIG. 6. Photomicrographs of crystal violet-stained peritoneal macrophages from *db/db* and *db/+* mice. Original magnification $\times 40$. **A:** Diabetic macrophages cultured for 40 h without LPS. **B:** Nondiabetic macrophages cultured for 40 h without LPS. **C:** Diabetic macrophages cultured for 40 h, 24 h after LPS (1 $\mu\text{g/ml}$) stimulation. **D:** Nondiabetic macrophages cultured for 40 h, 24 h after LPS (1 $\mu\text{g/ml}$) stimulation.

DISCUSSION

Both type 1 and type 2 diabetes are often complicated by increased susceptibility to infection, impaired wound healing, accelerated atherosclerosis, and other inflammatory or degenerative manifestations. A likely common denominator in all these abnormalities is the dysfunction of the macrophage system. Numerous phenotypic abnormalities, including altered metabolism (9–13), aberrations in chemotactic response (12–14), phagocytosis (12,15,16), antigen presentation (17–19), receptor functions (20,21), and cytokine release (18,22–27) have indeed been found in peripheral blood mononuclear cells from both type 1 and type 2 diabetic patients. On the other hand, data on the function of tissue macrophages in human diabetes are not available. We have therefore investigated macrophages in a murine model of type 2 diabetes and found that they release more NO and less of the typical macrophage cytokines (TNF- α and IL-1 β) and VEGF upon stimulation *in vitro* than the cells from phenotypically normal mice of the same strain. Diminished cytokine and growth factor release from *db/db*

macrophages may not represent a single defect in LPS-specific pathways because the amount of IL-1 β in diabetic cultures was also reduced when stimulants other than LPS were applied.

The histological appearance of wounds in *db/db* mice with poor cellularity, decreased angiogenesis, and significant delay in granulation tissue formation and re-epithelization (28) resembles what has been described in macrophage-depleted animals (29). Leibovich and Ross (29) proposed that the lack of macrophages led to the unavailability of soluble factors (apparently equivalent to various cytokines and growth factors discovered since), which consequently caused impaired tissue repair. Accordingly, the expression of IGF-I mRNA, as well as the production of the corresponding peptide, are significantly delayed and reduced in the wounds of *db/db* mice (30). Furthermore, topical application of platelet-derived growth factor and basic fibroblast growth factor, both known to be secreted by macrophages, improves the repair process in these animals (28). In addition, the immunoreactivity and amount of transforming growth factor- β 1 has been found to

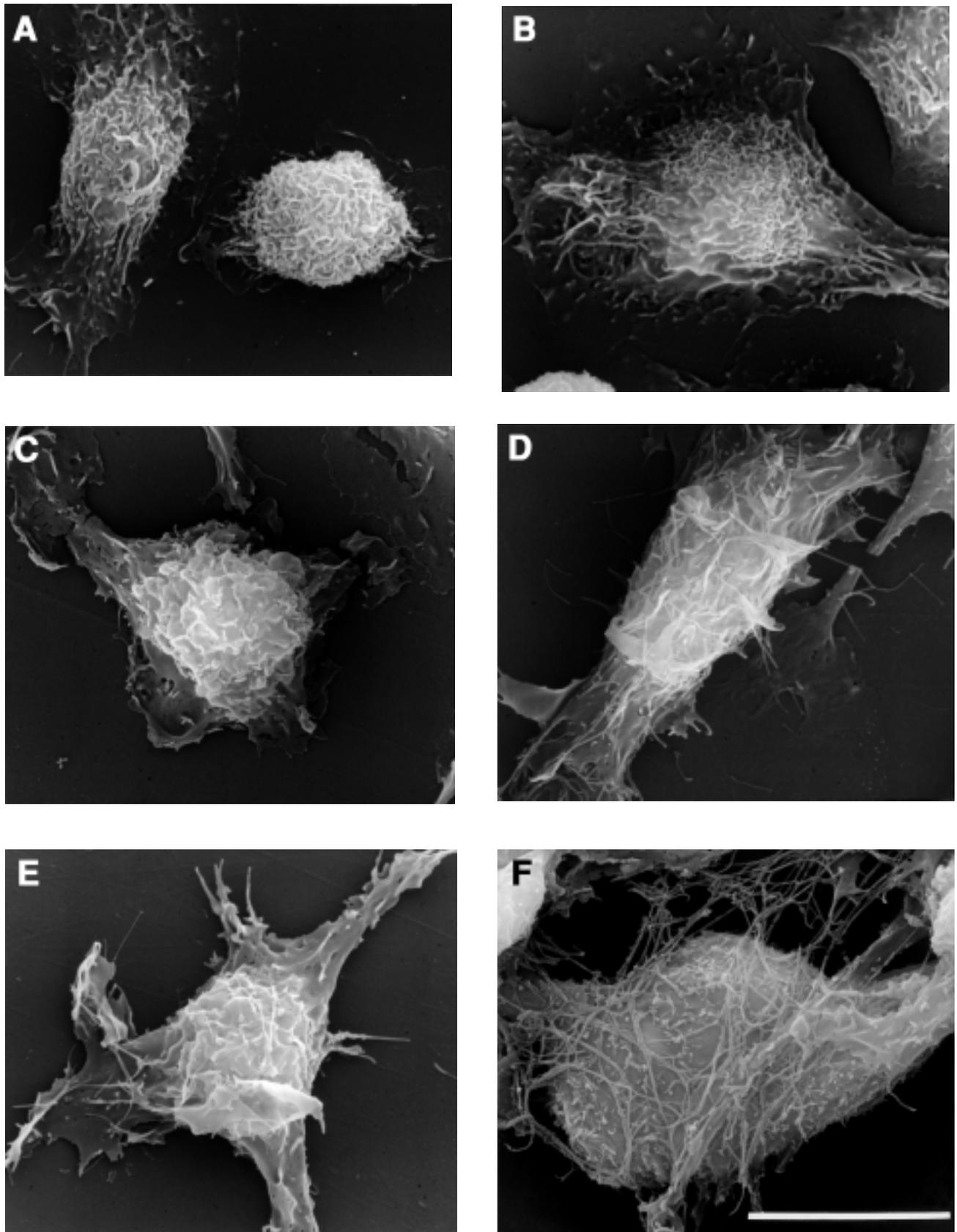


FIG. 7. Scanning electron micrographs of cultured peritoneal macrophages from *db/db* and *db/+* mice. **A:** Diabetic macrophages cultured for 2 h without LPS. **B:** Nondiabetic macrophages cultured for 2 h without LPS. **C:** Diabetic macrophages cultured for 24 h without LPS. **D:** Nondiabetic macrophages cultured for 24 h without LPS. **E:** Diabetic macrophages cultured for 24 h with LPS (1 µg/ml). **F:** Nondiabetic macrophages cultured for 24 h with LPS (1 µg/ml). Scale mark: 10 µm.

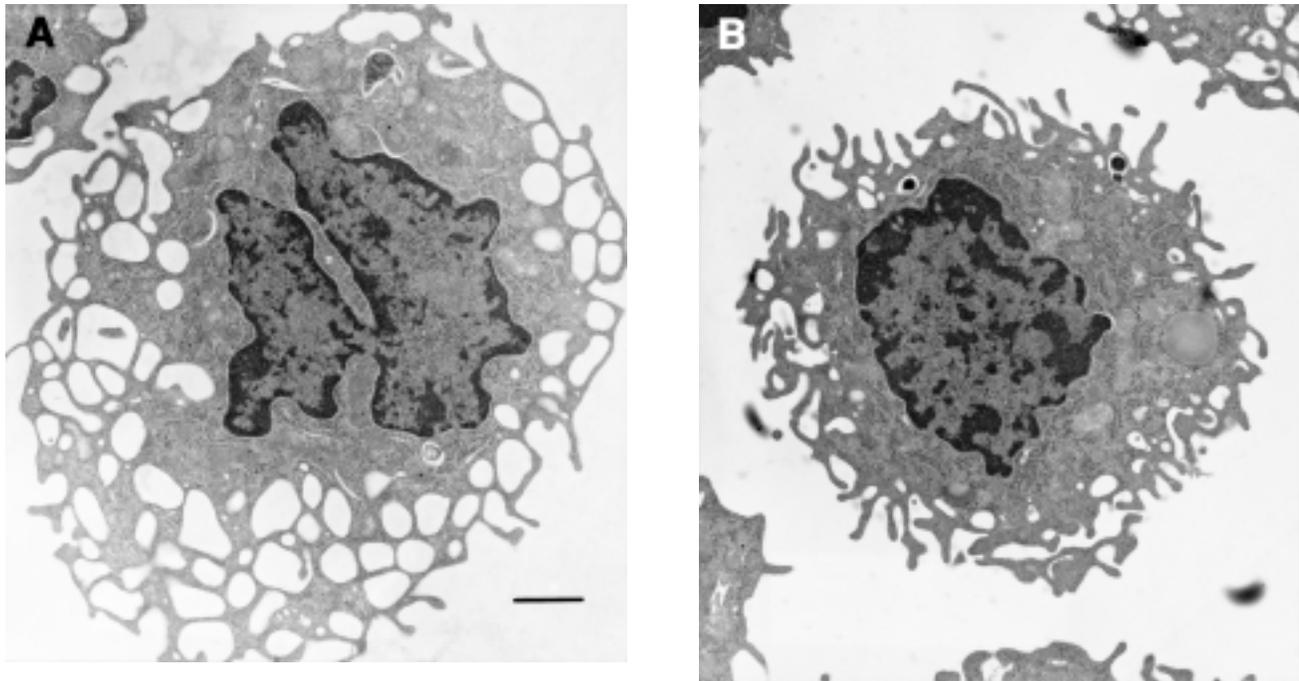


FIG. 8. Transmission electron micrographs of peritoneal macrophages from *db/db* and *db/+* mice immediately after harvesting. **A:** Diabetic macrophage. **B:** Nondiabetic macrophage. Scale mark: 1 μ m.

be decreased in ulcers of diabetic humans compared with that seen in diabetic and normal skin (31). The general concept of the significance of macrophage products has also been highlighted by successful therapy with growth factors in both animal and human diabetic wounds (28,32–34).

In *db/db* mice, the development of diabetes is coupled with the genetically conditional absence of the leptin receptor functional isoform (35,36), which is also found to be expressed in macrophages. Pre-exposure to leptin has a stimulatory effect on the LPS-induced release of TNF- α , IL-6, and IL-12 from cultured murine macrophages (but not IL-1 β and IL-10), and the *in vivo* cytokine response to LPS in the diabetic rat with leptin receptor deficiency is significantly reduced (4). However, the absence of a functional leptin receptor (and/or associated hyperleptinemia and leptin resistance) can hardly account for complications such as impaired wound healing in diabetes because this complication is not only a distinctive feature of *db/db* mice, but is also characteristic of other forms and animal models of the disease in which the leptin receptor is not defective. Regardless of the pathogenesis of diabetes, the diabetic state itself leads to changes in carbohydrate and lipid metabolism, hormonal balance, osmolarity, etc.—each of which could possibly affect the normal functioning of macrophages.

Hyperglycemic levels of glucose were shown to inhibit the release of IL-1 from LPS-stimulated macrophage-like RAW 264.7 cells and to stimulate NO production (37). It has also been reported that elevated glucose levels cause enhanced expression of inducible NO synthase (iNOS) and NO release in LPS plus IFN- γ -stimulated murine mesangial cells and the RAW 264.7 cell line (38).

Apart from the acute effects of high glucose concentrations, a common consequence of long-standing hyperglycemia is the

formation of advanced glycation end products (AGEs), i.e., nonenzymatic cross-links of biomolecules with monosaccharide derivatives. Macrophages, a primary scavenger cell type, possess a number of receptor species capable of binding and internalizing AGE-modified substances for which the further intracellular fate remains unknown. It seems that AGEs tend to accumulate in macrophages (39) and to be resistant to degradation (40), which in turn might compromise normal cellular functioning. Modification of the extracellular matrix proteins by conjugation with AGE could also disturb the interaction of immune cells—notably macrophages (11,41). Other factors, such as alterations in lipid metabolism, may also be involved (2).

We found that NO production was elevated in diabetic macrophages compared with normal macrophages, whereas there was no difference observed in the PMA-induced respiratory burst. Various reactive oxygen and nitrogen species released by activated macrophages have been postulated to exert strong cytotoxic and bacteriocidal effects, which could even affect the metabolic activity and vitality of the macrophages themselves (42,43). We do not know if this is related to the decrease in some macrophage functions, as demonstrated by the decreased release of cytokines. Several reports indicate upregulation of the iNOS pathway in the diabetic state (44,45). Nitrite/nitrate accumulation in wound fluid of streptozotocin-treated rats was significantly diminished (46). AGEs were reported to quench NO in tissues (47)—something that could possibly cause a relative decrease in the amounts of free NO *in situ* and, consequently, a compensatory hyperactivation of iNOS and absolute hyperproduction of NO by macrophages (31).

We observed extensive peripheral vacuolization in transmission electron micrographs of diabetic cells, which could either be a sign of a compensatory pinocytosis aiming to

restore diabetes-associated changes in the intracellular concentration of solutes or could reflect a generalized defect of membrane transport. Recently, a defect of exocytosis was implicated in the dysregulation of polymorphonuclear leukocyte antigen expression in human type 2 diabetes (48). It is possible that these findings are related to the diminished peptide release from macrophages in *db/db* mice that we report here, but further studies (also in human cells) are needed before we can understand the exact nature of the disturbance of the macrophage function in diabetes.

In conclusion, the *in vitro* release of TNF- α , IL-1 β , and VEGF is reduced in macrophages from *db/db* mice together with an increased production of NO. These results and the altered morphology of the macrophages indicate that macrophage function is severely impaired in diabetic mice of this type. Our observations are, in general, consistent with the reports based on studies of cytokine release from LPS-stimulated macrophages in other animal models of diabetes (1–3).

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