Cell-mediated Immunity in Chronically Diabetic Mice

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SUMMARY

A radioisotopic ear assay for delayed hypersensitivity (DH) to 2,4-dinitrofluorobenzene (DNFB) was used to investigate cellular immune function in chronic alloxan-diabetic C57Bl/6 mice. The specific migration of 5-(^{125}I)iodo-2′-deoxyuridine-labeled monocytes to the site of antigenic challenge was employed to quantitatively measure the DH response. Although both normal and diabetic sensitized mice had a significantly greater DH response than nonsensitized controls, the response of diabetic mice was severely attenuated (23% of normal).

The effect of the delayed hypersensitivity response on spleen size was also investigated. In the nonsensitized state the spleens of diabetic mice were approximately 30% smaller than the spleens of normal mice. After sensitization with DNFB, and up to the time of antigenic challenge, the spleens from both diabetic and normal mice increased 2.1-fold in weight. After challenge, however, the diabetic spleen maintained its prechallenged size while the normal spleen increased an additional 30% in weight.

When either sensitized normal or diabetic spleen cells were passively transferred to nonsensitized normal recipient mice, a significant DH response was evoked after challenge with DNFB. This response was of equal magnitude after transfer of either the sensitized diabetic or normal spleen cells. The DH response after the passive transfer of spleen cells from either normal or diabetic sensitized donors to diabetic recipients was very markedly attenuated and in fact questionably significant when compared with the nonspecific inflammatory response to DNFB.

These results indicate that an intact population of DNFB-sensitized cells exists in the diabetic, but that an inhibition of T-lymphocyte or monocyte activity is occurring during the secondary challenge. The chronic hypoinsulinemia, hyperglycemia, and catabolic metabolism of the alloxan-diabetic mouse does not qualitatively alter the immune cellular processes associated with primary sensitization but significantly attenuates the secondary response. DIABETES *29*:825–829, October 1980.

Early reports on cellular immunity in the diabetic indicated a transient deficiency. Brody and Merlie described a decreased glucose utilization and response to phytohemagglutinin (PHA) of diabetic lymphocytes.[1] Later, Bagdade et al. demonstrated a reduced rate of phagocytosis by polymorphonuclear leukocytes obtained from poorly controlled diabetic patients. In these patients the phagocytosis by polymorphonuclear leukocytes returned to normal levels after long-term insulin therapy.[2] No distinguishable differences in the response of the lymphocytes to PHA and *Candida albicans* were observed in vitro when lymphocytes from insulin-dependent diabetics were compared with lymphocytes from control subjects.[3]

More recently, the development of well-characterized, chronically diabetic mouse models has better defined the pathologic effects of diabetes mellitus on the cellular immune system. Work with genetically diabetic and obese C57Bl/KsJ db/db and C57Bl/6J ob/ob mouse strains has suggested an environmentally induced depression of cell-mediated immunity (CMI).[4] A discordance between in vivo compared with in vitro measurements of cell-mediated immunity has been observed.[5–8] In vivo experiments of allograft rejection, contact sensitivity, and granuloma formation demonstrate drastic reduction in the diabetic's immunologic responsiveness.[5,6] In contrast, when cellular-mediated immunity was tested in vitro, using immunocytes from the same strains, no attenuation of the response was observed.[7,8]

The severe attenuation of cellular immune functions both in vivo and in vitro has also been documented in chemically induced diabetic mice from various genetic back-

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grounds.[6,7,9,10,11] In these experiments, it was difficult to separate the effect of diabetes from the toxic effect of alloxan and streptozotocin because they were performed either concurrent with or 4 days after the administration of the diabetogenic agent.

The effects of chemically induced *chronic* insulinopenic diabetes on cellular immunity have not been extensively studied. We have therefore designed experiments in stabilized, chronically diabetic mice. A modification of the sensitive and quantitative radioisotopic ear assay[12,13] for delayed hypersensitivity to 2,4-dinitrofluorobenzene (DNFB, Sigma) was employed.

Passive transfer experiments were also designed to separate and measure the integrity of primary sensitization from secondary challenge in the diabetic environment. Sensitized spleen cells from normal and diabetic mice were transferred into diabetic recipients and, conversely, sensitized diabetic and normal spleen cells were transferred into normal recipients and the DH response measured. In these experiments a severe suppression of DH in the diabetic recipient was observed after transfer of either normal or diabetic sensitized spleen cells. On the other hand, a significant DH response was demonstrable in normal mice to which either normal or diabetic sensitized spleen cells were transferred. These observations are submitted as evidence that the diabetic milieu severely attenuates the cellular immune response associated with secondary challenge. The primary sensitization of the delayed cellular immune response does not appear to be qualitatively altered in the chronic alloxan-diabetic mouse.

MATERIALS AND METHODS

Animals. Chronic diabetes was induced in 8–12-wk-old C57Bl/6 male mice, weighing at least 23 g (Microbiological Associates, Walkerville, Maryland) with an intravenous injection (80 mg/kg) of alloxan monohydrate (J. T. Baker). The alloxan was diluted in phosphate-buffered saline, pH 7.4, to a concentration of 8.0 mg/ml and injected intravenously (i.v.) in a volume of 0.10 ml/10 g body wt. Alloxan-diabetic mice were age matched with normal mice. To minimize the toxic effects of alloxan, no mice were used prior to 4 wk after the alloxan injection.

Evaluation of the diabetic state. The blood glucose, glycosuria, and weight of all mice were monitored at 1 wk after alloxan treatment and at the time of delayed hypersensitivity assay.

Blood glucose was measured in 25-μl samples obtained from the periorbital sinus and diluted 1:5 in distilled water. The glucose concentration was determined enzymatically with a glucose analyzer (Yellow Springs Instruments). Blood glucose of normal mice did not exceed 200 mg/dl and averaged 134 ± 1.1 mg/dl (N = 177). The blood glucose of diabetic mice ranged from 300 to 700 mg/dl with a mean of 517 ± 6.4 mg/dl (N = 173).

Glycosuria was measured qualitatively on a negative to 4+ scale with diastix (Ameslo). All diabetic mice had 3+ or 4+ glycosuria whereas normal mice had no detectable glycosuria.

The body weights of diabetic mice stabilized approximately 2 wk after the alloxan injection at approximately 10% below that of age-matched control mice. During the 7-day period of the delayed hypersensitivity assay, sensitized diabetic mice averaged a weight loss of 4.6%, while normal sensitized mice lost approximately 3.0% of their body weight. Normal nonsensitized control mice, which did not receive cyclophosphamide, gained approximately 1.9% of their body weight over the same period, while diabetic nonsensitized mice lost 2.9%.

Antigen. The antigen, DNFB, diluted to 15 mg/ml in a 1:1 acetone:olive oil vehicle, was prepared fresh before each application.

Sensitization procedure. Mice to be sensitized received a 200-mg/kg subcutaneous injection of cyclophosphamide (Cytoxan, Mead/Johnson), in filtered distilled water, to augment delayed hypersensitivity responsiveness.[12,18] Two days after cyclophosphamide treatment, sensitization was initiated with the application of 25 μl of DNFB (0.6 mg) to the shaven/plucked skin of the back and abdomen, 15 μl to the back and 10 μl to the abdomen. An additional 25 μl of DNFB was applied the next day (day 3), 10 μl to the back and 15 μl to the abdomen. Sensitized spleen cells from either normal or diabetic mice for passive transfer were obtained 4 days (day 7) after the last sensitization with DNFB.

The delayed hypersensitivity (DH) assay. The DH assay was a modification of the radioisotopic ear technique described by Vadas and Miller et al.[12,14] Four days (day 7) after the last sensitization with DNFB, the mice were challenged with 3–5 μl of DNFB applied in a divided dose to the anterior and posterior surfaces of the left ear, respectively. Either no treatment or 3–5 μl of the vehicle solution was similarly applied to the right ear. Within 1–4 h, the mice were injected intraperitoneally (i.p.) with 0.1 ml of 10^{-3} M 5-fluorodeoxyuridine (F-Udr, Sigma) to inhibit de novo thymidine synthesis.[12,19] Exactly 20 min later an i.p. injection of 1.5 μCi of 5-(^{125}I)iodo-2′-deoxyuridine (^{125}I-Udr, Amersham, England, specific activity ≥ 5 Ci/mg) was administered. Both the F-Udr and ^{125}I-Udr were diluted in 0.85% saline, from stock solutions, immediately before the respective injections. Twenty-four hours after challenge, both ears were removed at the hairline and the ^{125}I counts/10 min of each ear were measured on a Beckman gamma spectrometer (^{125}I counting efficiency = 75.3%). The DH response was calculated from the cts/10 min in the challenged left ear minus the cts/10 min in the unchallenged right ear (L-R ear cts/10 min). The L-R ear cts/10 min are a measure of the migration of monocytic cells to the site of antigenic challenge.[12] By excluding the sensitization procedure, the background nonspecific inflammatory response to DNFB of nonsensitized normal and diabetic mice was measured. Spleens were removed and weighed to an accuracy of ±0.1 mg with a Mettler analytical balance immediately after the ears were excised.

Spleen cell suspensions. The preparations of sensitized spleen cells for passive transfer were obtained 4 days after the last sensitization with DNFB. The isolated spleens were weighed and carefully teased into cold balanced salt solution, pH 7.4, with curved forceps. The cell clumps were then dispersed by aspiration with a pasteur pipette. Spleen cells from either diabetic or normal mice were pooled and washed three times by centrifugation for 5 min at 1350 *g*. Pooled suspensions were adjusted to 1.0×10^8 viable nucleated cells/ml as determined by eosin dye exclusion. Spleen cell viability of cyclophosphamide-treated mice ranged from 50 to 75%.

Passive transfer of DH. The passive transfer of DH to nonsensitized recipients was performed by the i.v. injection of 5×10^7 viable spleen cells from DNFB-sensitized mice. Spleen cells from a normal (N) sensitized pool were transferred into both normal (N) and diabetic (D) recipients, resulting in N-N and N-D transfer groups. Likewise, diabetic-sensitized spleen cells were transferred into both normal and diabetic recipients, resulting in D-N and D-D groups. Within 1–2 h after the transfer of sensitized cells, 3–5 μl DNFB was applied to the left ear of the nonsensitized recipients and the delayed hypersensitivity response measured as previously described.

Histology. The individual excised ears were immediately fixed in 1.0 ml neutral formalin, pH 7.4, and the cts/10 min of each ear measured while in the fixative. One to two days later the ears were transferred into 0.1 M sodium cacodylate buffer, pH 7.4, and stored at 4°C until the time of paraffin embedding. Sections (2 μm) were stained with hematoxylin and eosin for light microscopic examination.

Statistical analysis. All data were grouped and represented by arithmetic mean (X), standard error of the mean (SEM), and group size (N). Statistical comparison of groups were performed by a Student's *t* test between means of unpaired data. The results of cell transfer studies represent a total of 12 individual experiments.

RESULTS

The DH response. The DH response of alloxan-diabetic mice, 1131 cts/10 min, was sixfold that of nonsensitized controls. While this represents a significant ($P < 0.001$) response, it was only 23% of the response of normal sensitized mice. The expression of DH by the alloxan-diabetic mice was therefore significantly ($P < 0.001$) attenuated (Figure 1).

The nonspecific inflammatory responses of nonsensitized normal and diabetic mice to DNFB, when combined, averaged 163 cts/10 min. This value represents less than 4% of the response of normal sensitized mice. No significant difference was observed between the responses of nonsensitized normal and diabetic mice.

Histopathology. The ears from chronic diabetic mice were thinner with a narrow atrophic cartilage plate, relatively few attenuated skeletal muscle fibers, a thin epidermis 1–2 cells in thickness, and thin, relatively scanty collagen fibers in the dermis. By comparison, the ears of the normal mice are approximately twice as thick, with a robust cartilage plate, relatively numerous and hypertrophied skeletal muscle fibers, an epidermis that is 3–4 cells thick, and relatively dense collagen fibers in the dermis.

There was marked edema and an intense infiltrate of mononuclear cells with vascular leukocyte margination in the sections of the challenged ear from normal sensitized mice. The attenuated DH response of the diabetic is readily appreciated upon histologic examination. In the diabetic response there was less edema and fewer mononuclear cells, corresponding to the differences in (left-right) ear counts. The normal nonsensitized ear shows essentially no infiltrate at 24 h.

Effect of DH on spleen size. Enlargement of the spleen was observed concurrent with the development of delayed hypersensitivity to DNFB in mice (Table 1). In the nonsensitized state the spleen from diabetic mice (51.8 ± 3.1 mg) weighed approximately 30% less than that from normal mice (72.7 ± 2.5 mg). This 30% smaller spleen size of diabetic mice was disproportionate with the 10% lower body weight. By the fourth day after sensitization (day 7), the normal spleen had enlarged 2.1-fold to a weight of 150.4 ± 8.2 mg. This change was paralleled by a 2.1-fold increase in the diabetic spleen to a weight of 109.7 ± 8.4 mg. The mice were challenged on day 7 and the spleen size measured 24 h later. The normal spleen enlarged an additional 30% to 199.6 ± 6.1 mg, while the diabetic spleen remained constant at 103.0 ± 8.7 mg. Thus, there was spleen hypertrophy in the diabetic and normal mice during the DNFB primary sensitization of an equal degree, but there was spleen

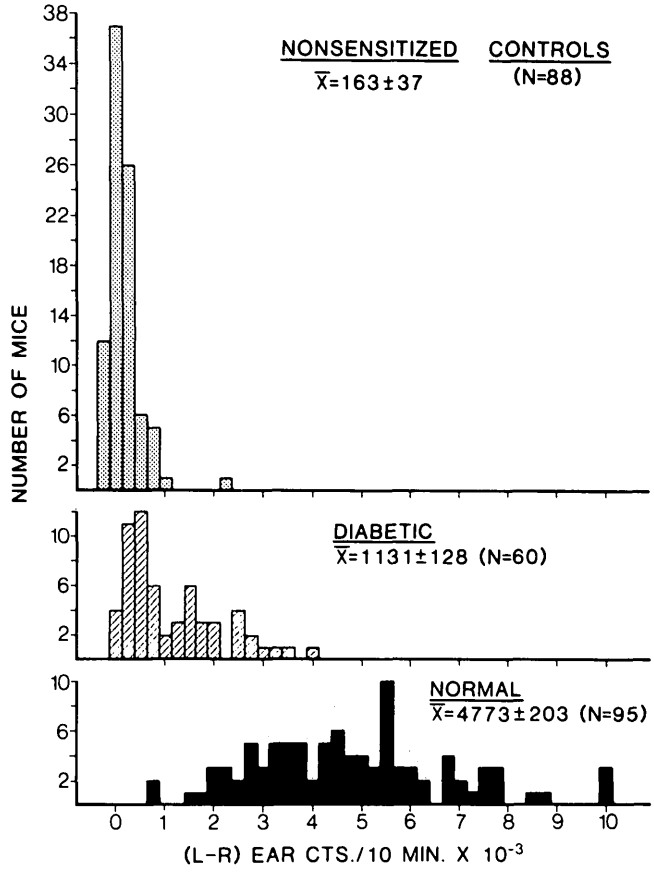

FIGURE 1. The effect of diabetes on the delayed hypersensitivity response to DFNB. The nonsensitized controls consisted of 29 diabetic and 59 normal mice. The response of normal and diabetic nonsensitized mice to DNFB was not significantly different (P = 0.65).

TABLE 1
The effect of diabetes on spleen weight during the delayed hypersensitivity response

	Normal	Diabetic
Day 0 Nonsensitized	72.7 ± 2.5 (N = 29)	51.8 ± 3.1 (N = 51)
Day 7 Sensitized	150.4 ± 8.2 (N = 19)	109.7 ± 8.4 (N = 17)
Day 8 Sensitized and challenged	199.6 ± 6.1 (N = 32)	103.0 ± 8.7 (N = 23)

Values shown are the mean weight of the spleens in mg ± SEM. (N = number of mice studied.)

hypertrophy only in the normal sensitized mice after DNFB challenge.

Passive transfer of DH. The passive transfer of DH to DNFB was studied in four experimental groups: (1) the transfer of sensitized spleen cells from normal mice to nonsensitized normal inbred recipient mice (N-N), (2) the transfer of sensitized spleen cells from chronic diabetic mice to nonsensitized normal inbred recipient mice (D-N), (3) the transfer of sensitized spleen cells from normal mice to nonsensitized chronic diabetic inbred recipient mice (N-D), and (4) the transfer of sensitized spleen cells from chronic diabetic mice to nonsensitized chronic diabetic inbred recipient mice (D-D).

A significant transfer of the DH response to DNFB was observed when normal mice were the recipients in either the N-N or D-N experiments (Table 2). The magnitude of the DH response was equal in the N-N (677 ± 69 cts/10 min) and D-N (626 ± 82 cts/10 min) transfer experiments. These responses were approximately fourfold greater than the nonspecific inflammatory response of nonsensitized controls ($P < 0.001$) (Table 2). Although the DH response of sensitized diabetic mice was 23% of that observed in normal sensitized mice, the DH response conferred in the D-N transfer was 93% of that conferred in the N-N transfer. It follows that the capacity of the challenged diabetic lymphocytes to mount a DH response when transferred to the normal environment is markedly augmented.

This is supported by the very slight DH response observed in the transfer of either normal (N-D) or diabetic (D-D) sensitized spleen cells to chronic diabetic recipients (Table 2). The N-D transfer response (284 ± 39 cts/10 min) and the D-D transfer response (228 ± 40 cts/10 min) were approximately one-third that observed when normal mice were the recipients. The diabetic recipient was able to host, at best, a very slight passive transfer of the DH response that was marginally significant when compared with the nonspecific inflammatory response to DNFB (Table 2). These observations are submitted as evidence that the diabetic recipient compared with the normal recipient has a markedly attenuated capacity to host the transfer of DH.

TABLE 2
Effect of diabetes on the passive transfer of delayed hypersensitivity

Experimental groups	DH response	(N)
Nonsensitized		
Normal	175 ± 51	(59)
Diabetic	137 ± 45	(29)
Sensitized		
Normal	4773 ± 203	(95)
Diabetic	1131 ± 128	(60)
Transfer to normal		
N-N	677 ± 69	(60)
D-N	626 ± 83	(30)
Transfer to diabetic		
N-D	284 ± 39	(40)
D-D	228 ± 40	(23)

The values are the mean cts/10 min of the L ear–the R ear ± SEM. (N) is the number of mice per group. The DH response of the nonsensitized group of mice represents the nonspecific inflammatory response to DNFB. N-N indicates the group of mice in which sensitized spleen cells from normal mice were transferred to nonsensitized normal mice. D-N indicates the group of mice in which sensitized spleen cells from diabetic mice were transferred to nonsensitized normal mice. N-D indicates the group of mice in which sensitized spleen cells from normal mice were transferred to nonsensitized diabetic mice. D-D indicates the group of mice in which sensitized spleen cells from diabetic mice were transferred to nonsensitized diabetic mice.

DISCUSSION

The attenuated DH response to DNFB of the diabetic mice observed in our studies is consistent with other studies on the suppression of granuloma formation, contact sensitivity (as measured by ear swelling), delayed footpad swelling, and allograft rejection in alloxan- and streptozotocin-diabetic mice.[6,7,9–11] Of interest is the fact that in the majority of the other studies, and in our study, the diabetic mouse was capable of expressing some response, although minimal.

The sensitivity and precision of the radioisotopic ear assay[12,14] was combined with the passive transfer of DH to better identify the mechanism(s) involved in this diabetic immunodeficiency. Lymphocytes from normal sensitized mice were challenged after transfer into diabetic recipients, and, conversely, sensitized diabetic lymphocytes were challenged after transfer into normal recipients. In this manner, the effect of the diabetic environment on two temporally spaced components of DH, primary sensitization and secondary challenge, were analyzed. During primary sensitization the initial processing of antigen and the resulting expansion of T-lymphocyte populations with specificity for DNFB takes place. Secondary challenge requires blast transformation of these sensitized lymphoctes, production and recognition of lymphocyte-monocyte interactions, and the final replication and migration of labeled monocytes to the site of antigenic challenge. Theoretically, the lack of insulin and/or the corresponding alteration of metabolism in the diabetic could inhibit any of these cellular processes.

The results of the passive transfer experiments describe an immune state in the diabetic where primary sensitization to DNFB is intact, but where the expression of delayed hypersensitivity is not. Direct comparison of the N-N and D-N transfer responses was unable to distinguish a difference between the two populations of lymphocytes when placed in a normal environment. While the diabetic spleen is 30% smaller than normal, and might therefore result in a slightly suppressed DH, this is apparently not the cause of the diabetic immunodeficiency. Analysis of both the N-D and D-D transfer groups indicates that a population of DNFB-sensitized lymphocytes, whether from normal or diabetic donors, cannot elicit a DH reaction when challenged in the diabetic environment. The proliferative response occurring in the spleen is consistent with an impaired immune response during secondary challenge in the diabetic. During the period of primary sensitization, both the normal and diabetic spleens experienced an equal relative increase in size. After secondary challenge, however, the normal spleen increased a significant 30% (49 mg), while the diabetic spleen remained at its prechallenged size.

It is suggested that primary sensitization to DNFB is intact in the diabetic and that an inhibition of T-lymphocyte or monocyte activity results during secondary challenge. The exact nature, or population, of cells affected is not readily determined from these experiments. Whitesell et al. re-

ported that thymocyte activation is accompanied by a marked increase in glucose transport.[15] Whether or not this activation is under the regulation of insulin, or related to the emergence of insulin receptors observed on stimulated normal lymphocytes, is not clear.[16]

The chronically diabetic mice in these experiments have insulinopenia, hyperglycemia, and a catabolic metabolism. Our results indicate that one, or a combination, of these factors induces an immunodeficiency in vivo characterized by impaired T-lymphocyte or monocyte activity during secondary challenge. One likely factor is insulinopenia. In preliminary experiments, a significant enhancement of the attenuated DH response has been observed in insulin-treated chronic diabetic mice. In addition, an attenuation of the DH response was observed when normal animals were made insulinopenic after the injection of insulin antisera.[17] Although this preliminary evidence implicates insulin as an important factor in this observed immunodeficiency, the degree to which hyperglycemic and catabolic factors also contribute remains to be tested. It is proposed that this immunodeficiency resulted in the marked attenuation of the DH response observed in the passive transfer to alloxan-diabetic recipients.

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