Mitogen Responsiveness of Lymphocytes From the BB/W Rat

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SUMMARY

The response of BB diabetes-prone (DP) and W-line non-diabetes-prone rats to the T-cell mitogen concanavalin A (ConA) was measured. The W line was a good responder to ConA, whereas the DP was relatively unresponsive. This unresponsiveness could not be reversed with exogenous interleukin 2 (IL-2). The response of DP rats was enhanced by removing adherent cells. To directly test the response of BB T-cells, they were isolated by flow sorting. These experiments demonstrated that BB T-cells could mount a normal ConA response. The normal function of isolated BB T-cells suggested that they were under suppression. Suppressor activity could not be found in the $OX8^+$ population but was found in the DP-adherent cell population. Adherent cells from the W line were not suppressive at the concentrations used. These results showed that the decreased mitogen responsiveness of BB T-cells was not due to an intrinsic T-cell abnormality but was due, in part, to suppression by adherent cells. DIABETES 1986; 36:513–16.

The BB/W (Bio-Breeding/Worcester) rat develops spontaneous autoimmune diabetes at 60–90 days of age. The diabetic animals are ketoacidotic and insulin dependent.[1] Histologic examination of the pancreas from diabetic animals shows insulitis.[2] The presence of insulitis suggests an autoimmune pathogenesis for the diabetes. This hypothesis is supported by the observation that diabetes can be transferred by concanavalin A (ConA)-stimulated spleen cells obtained from acute diabetic rats.[3] The disease can be prevented by neonatal thymectomy,[4] antilymphocyte serum treatment,[5] cyclosporin A,[6,7] bone marrow transplantation,[8] total body irradiation,[9] or transfusion of peripheral blood T-lymphocytes from non-diabetes-prone (W line) animals into diabetes-prone (DP) recipients.[10]

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Although these rats develop an autoimmune disease, paradoxically, they are immune deficient. These animals display severe T-lymphopenia[11–13] and decreased mitogen and mixed lymphocyte responses.[14–17] It has recently been shown that macrophages act as suppressor elements in the mitogen responsiveness of the BB rat.[18] In these experiments, we studied the response of DP and W-line spleen cells to the T-cell mitogen ConA. As expected, DP spleen cells had markedly suppressed ConA responses. However, when DP T-cells were purified, they exhibited normal ConA responses, directly demonstrating that DP T-cells are not intrinsically abnormal but are under suppression.

MATERIALS AND METHODS

Rats. BB/W rats 8–12 wk of age were obtained from the breeding colony maintained at the University of Massachusetts Medical School. DP and W-line rats were used in these experiments. DP rats develop spontaneous insulin-dependent diabetes at 60–90 days of age at a frequency of 50–60%. The W line is a subline of the BB/W that has an incidence of diabetes of 1% through 17 generations. Rats were housed under standard laboratory conditions.

Cells. Spleen cell suspensions were prepared by teasing apart spleens in minimum essential medium [(MEM) Gibco, Grand Island, New York] supplemented with 5% fetal bovine serum [(FBS) Gibco] and by filtering them through nylon mesh. Red blood cells were lysed with Tris-buffered ammonium chloride (0.015 M Tris-0.73% ammonium chloride, pH 7.4). The spleen cells were sedimented through FBS, washed twice with MEM-FBS, and adjusted to the appropriate cell concentration.

Adherent cells were depleted from spleen cell populations by incubation at 37°C for 2 h on tissue culture-treated plates at a density of 5×10^7 cells/100-mm plate. Nonadherent cells were then washed off. Adherent cells were prepared by washing the plates with 0.5 mM EGTA in Ca-Mg-free Hanks' balanced salt solution (EGTA-HBSS). Five milliliters of EGTA-HBSS was then added, and the plates were incubated at 37°C for 5 min. Adherent cells were removed by pipetting and were washed in RPMI-FBS. Adherent cells from

TABLE 1
ConA responses

	W line		Diabetes prone		
	ConA	RPMI	ConA	RPMI	Lewis
Whole spleen	102,770 ± 7,484* (25)	1,901 ± 1,102 (25)	4,854 ± 1,377 (28)†	2,075 ± 337 (28)	126,000 ± 6,000 (2)
Nonadherent cells	109,598 ± 8,745 (21)	4,208 ± 1,227 (21)	29,885 ± 5,488 (21)†‡	2,280 ± 125 (21)	
$OX19^+$	126,819 ± 11,906 (7)	2,003 ± 1,352 (6)	118,565 ± 4,109 (8)	160 (1)	
$OX19^-$	2,106 ± 816 (5)	796 ± 710 (4)	324 ± 89 (4)	1,246 ± 805 (4)	
OX19-labeled not sorted	121,514 ± 20,289 (4)	3,581 ± 1,779 (4)	5,696 ± 4,149 (4)	507 ± 387 (4)	

*Mean thymidine incorporation ± SEM. In parentheses are the number of separate experiments.
†P-value for comparison between W line and DP calculated by Student's *t*-test is <0.0001.
‡P-value for comparison of DP unseparated and nonadherent populations is <0.0001.

DP and W line prepared in this manner contained ~30% nonspecific esterase-positive cells.

Antibodies. OX8, a monoclonal antibody that reacts with T-cytotoxic-suppressor cells and NK-cells,[19,20] and OX19, a monoclonal antibody that reacts with T-cells,[21] were derived from the supernatants of the respective hybridomas (gift of Dr. A. Williams). Fl-$F(ab')_2$ goat anti-mouse IgG that does not cross-react with rat IgG was obtained from Cappel Laboratories, West Chester, Pennsylvania.

Cell staining. Mononuclear cells at a concentration of 3×10^7/ml were incubated with OX19 or, in some experiments, OX8 for 30 min at 4°C. The cells were washed 3 times and were then stained with 80 μg/ml of fluoresceinated goat anti-mouse IgG for 30 min at 4°C. The cells were washed 3 times and were resuspended at a final concentration of 5×10^6/ml.

Cell sorting. Cells were fractionated into positive and negative populations with a FACS 440 (Becton-Dickinson Immunocytometry Systems, Mountain View, California). Cells were separated into $OX19^-$ and $OX19^+$ and $OX8^-$ and $OX8^+$ subpopulations using aseptic technique at a flow rate of 2500–3000 cells/s. In each experiment, analysis of the negative cell fraction showed <1% positive cells. The positive fractions were 96–99% positive.

Mitogen stimulation. Unfractionated or fractionated cells were stimulated with the T-cell mitogen ConA at a final concentration of 2 μg/ml. Dose-response curves at concentrations of ConA from 0.1 to 10 μg/ml were generated for both DP and W-line rats, with 2 μg/ml being the optimum for each group.

The effect of adding exogenous interleukin 2 (IL-2) to DP spleen cell cultures was determined by adding IL-2 at a final concentration of 78 and 26 U/ml. The IL-2 source was a ConA supernatant from Lewis rats with an IL-2 concentration of 150 U/ml.

Cells in RPMI 1640, 10% FBS, 1×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 10 mM Hepes, and penicillin-streptomycin at a concentration of 5×10^5/ml were cultured in triplicate in flat-bottomed microtiter plates (Falcon, Oxnard, California) in 5% CO_2-95% air at 37°C for 66 h. Cells were then pulsed with 0.5 μCi tritiated thymidine (New England Nuclear, Boston, Massachusetts). The cells were harvested 6 h later onto glass fiber filters. The filters were air-dried, placed into scintillation fluid, and counted.

IL-2 production assay. The ability of spleen cells to produce IL-2 was assessed by culturing unfractionated or nonadherent cells at a concentration of 2×10^6/ml for 48 h in medium containing 2 μg/ml ConA. Supernatant fluids were tested for IL-2 activity with the IL-2-dependent cytolytic T-cell line CTLL-2. One unit of IL-2 was defined as the amount needed to induce half-maximal proliferation of CTLL-2.

Suppressor cell assays. In this variation of the ConA assay, 1×10^5 nonadherent W-line lymphocytes were used as responder cells. Adherent cells from W line or DP were added back to the W-line cells at a final concentration of 50 or 25%. The percent of suppression was calculated as follows:

$$\frac{1 - \text{W-line cells + adherent cells} \times 100}{\text{W-line cells}}$$

In the OX8 suppressor assay, OX8 cells at a final concentration of 50% were added to W-line nonadherent cells. The effect of indomethacin on the suppression by DP adherent cells was assessed by adding 5×10^{-6} M indomethacin to the cultures.

TABLE 2
Addition of exogenous IL-2 does not enhance ConA responsiveness of DP spleen cells*

	Thymidine incorporation
DP + 2 μg/ml ConA	5931
DP + 2 μg/ml ConA + 26 U/ml IL-2	3299
DP + 2 μg/ml ConA + 78 U/ml IL-2	1365
DP, no mitogen	939

*The data represent the mean thymidine incorporation in quadruplicate wells in a single representative experiment.

TABLE 3
IL-2 secretion

	W line	Diabetes prone
Unseparated	34 ± 6* U/ml (4)	4.6 ± 0.7 U/ml (6)†
Nonadherent	46 ± 5 U/ml (5)	7.3 ± 2 U/ml (5)†

*Mean ± SEM. In parentheses are the number of separate experiments.
†P-value for comparison between W line and DP calculated by Student's *t*-test is <0.02.

TABLE 4
Attempted suppression of the ConA response with DP OX8+ cytotoxic/suppressor cells*

	Thymidine incorporation
W nonadherent cells	169,103
W + 50% DP OX8	194,479
W + 25% DP adherent cells	34,057
W + 50% DP adherent cells	11,231
W, no mitogen	9,345

*The data represent the mean thymidine incorporation in triplicate wells in a single experiment.

RESULTS

Mitogen stimulation. W-line rats had a vigorous mitogenic response to ConA (Table 1), comparable in magnitude to a good ConA responder, such as Lewis rats. This response was not appreciably enhanced when adherent cells were depleted. DP rats were poor responders to ConA. Although this response was enhanced about sixfold when spleen cell populations were depleted of adherent cells, it still remained far below that of W-line cells. In four rats, the starting spleen cell population contained 14 ± 1% OX19+ T-cells. The nonadherent population contained 12 ± 1% OX19+ T-cells. Hence, enrichment of T-cells could not account for the enhanced mitogenesis. The response to ConA was not enhanced by adding exogenous Lewis rat IL-2 to the cultures at concentrations of 78 and 26 U/ml (Table 2).

To examine the activity of T-cell populations when removed from possible non-T-cell-suppressor elements, T-cells were purified by flow sorting. Purified T-cells from W-line and DP animals responded equally well to ConA (Table 1).

IL-2 production. The IL-2 production by DP unseparated and by nonadherent cells was significantly less than that of W-line cells (Table 3). For each group the IL-2 production by nonadherent cells was slightly enhanced compared with unseparated cells.

Suppressor cell activity. Because we observed that purified T-cells from DP animals made a vigorous response to ConA, we tried to find a possible suppressor element for the mitogenic response. In our first experiment, we added isolated DP cytotoxic/suppressor T-cells (OX8+) to W-line nonadherent cells. The OX8 cells did not suppress the ConA response (Table 4). When DP adherent cells were added to W-line responder cells, they markedly suppressed the ConA response (Tables 4 and 5). W-line adherent cells at the concentration used did not have such suppressive activity and, in fact, slightly enhanced the ConA response. The suppressive activity of DP adherent cells could be decreased by adding the prostaglandin antagonist indomethacin to the cultures. As shown in Table 5, appreciable suppressive activity remained, even with indomethacin. The suppressive activity could not be decreased with catalase, a peroxide antagonist (data not shown).

DISCUSSION

In this study, we have demonstrated that DP rats are relatively unresponsive to the T-cell mitogen ConA. This unresponsiveness appears to be due, in part, to suppression by an adherent cell that is active in DP spleen cell populations but not in W-line populations. Isolated T-cells from DP respond vigorously to ConA, directly demonstrating that they are functionally intact. Note that thoracic duct lymphocytes, which do not have large numbers of adherent cells, are hyporesponsive,[13] suggesting that adherent suppressor cells cannot account for the complete deficit in T-cell function. The inability of exogenous IL-2 to correct T-cell hyporesponsiveness has been previously reported by Naji et al.[13] and Bellgrau et al.[17]

In this study, we isolated OX19+ T-cells by flow sorting. OX19 is a costimulator in mitogenic responses but by itself is not mitogenic,[22] and, hence, the binding of OX19 to BB lymphocytes should not have influenced our results.

The hyporesponsiveness of BB lymphocytes to ConA has been reported before.[14–16,18] The study of Prud'homme et al.[18] showed that adherent cells suppressed the lectin response and that these cultures had reduced amounts of IL-2. Another study failed to find suppressive spleen cells and reported "normal" IL-2 production by BB spleen cells.[15] Our data, which document the presence of suppressor cells and only low production of IL-2, confirm the study of Prud'homme et

TABLE 5
*ConA responses are inhibited by DP adherent cells**

Exp.	W nonadherent cells	Plus W adherent cells		Plus DP adherent cells		DP adherent cells plus indomethacin	
		1 × 10^5	5 × 10^4	1 × 10^5	5 × 10^4	1 × 10^5	5 × 10^4
		Inhibition, %					
1	105,596	129,206 (−22)	114,905 (−9)	456 (99)	3,647 (97)		
2	109,386	156,402 (−43)	144,216 (−32)	56,912 (48)	55,027 (50)	81,464 (26)	140,363 (−28)
3	119,689	142,646 (−19)	158,682 (−33)	28,034 (77)	102,510 (14)	42,341 (65)	110,187 (8)
4	142,624	152,463 (−7)	171,192 (−20)	34,095 (76)	132,629 (7)	63,491 (55)	148,011 (−4)
5	191,187			55,353 (71)	94,064 (51)	89,844 (53)	118,890 (38)
6	109,307	122,594 (−12)	124,302 (−14)	59,648 (45)	99,319 (9)		
7	88,843	81,150 (9)	100,650 (−13)				
		−16 ± 7	−20 ± 7	69 ± 8†	38 ± 14†	50 ± 4‡	4 ± 14‡

*In this experiment, 1 × 10^5 W-line nonadherent cells were stimulated with ConA. 1 × 10^5 of 5 × 10^4 W-line or DP adherent cells were added to the cultures. The % inhibition was calculated as outlined in the MATERIALS AND METHODS section. In some experiments, indomethacin plus DP adherent cells were added to the W-line responders.
†P-value for comparison between W line and DP calculated by Student's *t*-test is <0.0001.
‡P-value for DP adherent cells and DP adherent cells plus indomethacin is 0.14.

al.[18] Our study extends Prud'homme's study by demonstrating that when T-cells from DP rats are isolated, they proliferate normally to a ConA stimulus. This proves that DP T-cells can process activation signals, produce IL-2, express IL-2 receptors, and process IL-2 signals. Apparently, in a mixed spleen cell population, DP T-cells are under so much suppression that they cannot proliferate normally. Suppression appears to be dominant, and the paucity of T-cells in DP spleens probably contributes to the hyporesponsiveness. If DP T-cells comprise only 25% of those present in W-line spleen, one might expect a 4-fold but not the observed 21-fold decrease in the ConA response.

Normal T-cell proliferation depends on the presence of IL-1, a macrophage product, and IL-2, a T-cell product. Prud'homme[18] has shown that there is no defect in IL-1 production by BB macrophages. Similarly, macrophages in rats with adjuvant arthritis that exhibit T-cell deficits similar to those seen in DP rats showed normal IL-1 production.[23] The deficit in IL-2 production by DP spleen cells could be due to an inability to secrete IL-2 or to an inability to process the ConA activation signal resulting in IL-2 production. The ability of isolated DP T-cells to proliferate normally in response to ConA rules out these IL-2 defects. Deficiencies in IL-2 production also do not explain the hyporesponsiveness of DP T-cells because exogenous IL-2 does not enhance their ConA response. The adherent suppressor cells in DP rats probably interfere with early activation signals.

The inhibition of adherent cell suppression by indomethacin did not reach significance, although indomethacin always enhanced mitogenesis. This suggests that while prostaglandins might inhibit mitogenesis, another mechanism of suppression may be present. Note that the suppressive activity of DP adherent cells may make it difficult to generate in vitro immune responses.

An important issue in the BB rat is the role of the peripheral T-cell abnormalities in the pathogenesis of BB diabetes. Although it is difficult to correlate T-cell hyporesponsiveness with autoimmunity, this T-cell defect may be a product of abnormal immunoregulation, which could allow the development of organ-specific immunity.

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