PREVENTION OF DIABETES IN BIOBREEDING/WORCESTER RATS WITH MONOCLONAL ANTIBODIES THAT RECOGNIZE T LYMPHOCYTES OR NATURAL KILLER CELLS

By A. A. LIKE, C. A. BIRON, E. J. WERINGER, K. BYMAN, E. SROCZYNSKI, and D. L. GUBERSKI

From the Department of Pathology, University of Massachusetts Medical School, Worcester, Massachusetts 01605

The BioBreeding rat develops a spontaneous syndrome resembling human type I diabetes mellitus. Salient features include abrupt onset of insulin dependent, ketosis-prone diabetes between 60–120 d of age (1); lymphocytic insulitis with virtually complete destruction of pancreatic β cells (2, 3); and genetic predisposition and occurrence of hyperglycemia in the majority of inbred BioBreeding/Worcester (BB/Wor)[1] animals (4).

Support for an immune pathogenesis of BB/Wor diabetes is derived from the following lines of evidence: lymphocytic insulitis before and during the acute phase of hyperglycemia (1–3); ability to adoptively transfer diabetes with injections of Con-A stimulated diabetic spleen cells (5, 6); MHC-linked susceptibility to diabetes (7); apparent presence of antibodies against islet cell surface molecules in diabetic and diabetes-prone animals (8); prevention of diabetes by neonatal thymectomy and immune suppression (9–14); prevention of diabetes by immune enhancement measures that correct the rats' immune incompetence (15–18).

The precise phenotypic identification of the effector cells that mediate final destruction of the pancreatic β cells has not been accomplished. Although lymphocytes and macrophages infiltrate the pancreatic islets, and Ia^+ macrophages are numerous (2, 19, 20), the functional role and specificity of these inflammatory cells is uncertain, since animals protected from diabetes by most immune-suppressive measures (9–11, 13) frequently show histologic evidence of lymphocytic insulitis despite the presence of normal plasma glucose levels. The questions raised by the latter morphologic observations encouraged us to design experiments for the treatment of diabetes-prone BB/Wor rats using mAbs that react with antigens expressed by rat lymphocyte subsets. Our experiments

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This manuscript is dedicated to George F. Cahill, Jr. in recognition of his landmark contributions to our understanding of the pathogenesis of diabetes mellitus.

[1] *Abbreviations used in this paper:* BB/Wor, BioBreeding/Worcester; BFA, Bonferonni adjustment; DB-PRN, diabetes-prone; DB-RES, diabetes-resistant; EAE, experimental allergic encephalomyelitis; LSD, least significant difference; SNK, Student-Newman-Keuls; TCS, tissue culture supernatant.

TABLE I
mAbs Used in This Study

Hybridoma number	IgG class	Specificity
W3/13	IgG1	T cells, thymocytes, polymorphs, plasma cells, NK cells, brain
W3/25 (CD4)	IgG1	Helper/inducer cells (H/I), macrophages, thymocytes
OX35 (CD4)	IgG2a	H/I cells, macrophages, thymocytes. Does not compete with W3/25 or OX38
OX38 (CD4)	IgG2a	H/I cells, macrophages, thymocytes. Competes with W3/25 but not with OX35
OX8 (CD8)	IgG1	Cytotoxic/suppressor cells, NK, thymocytes
OX19 (CD5)	IgG1	T cells, thymocytes
OX52	IgG1	T cells, thymocytes. Does not compete with OX19
OX6	IgG1	Ia-A, nonpolymorphic
OX17	IgG1	Ia-E, nonpolymorphic
3JP	IgG2a	Ia-A, nonpolymorphic

contained two principal objectives: first, to determine whether injections of mAbs would in fact be useful for the prevention of BB/Wor diabetes; second, if we were successful in achieving our first objective, to identify the lymphocyte subset(s) that mediate pancreatic β cell destruction.

We report here that (*a*) phenotypic OX19-reactive lymphocytes (all T cells) and OX8-cells (cytotoxic/suppressor and NK cells) were selectively depleted from adult BB/Wor rats by prolonged injections of tissue culture supernatants (TCS) containing OX19 or OX8 mAbs; (*b*) NK lytic activity was significantly reduced in OX8-depleted animals; (*c*) rats treated with either OX8 or OX19 TCS were protected against the occurrence of diabetes. We conclude that the subsets of rat lymphocytes that express OX8 and OX19 surface antigens participate in BB/Wor pancreatic β cell destruction.

Materials and Methods

Animals. Intact litters of diabetes-prone (DB-PRN) BB/Wor rats, 25–35 d of age, were obtained from the breeding colony maintained at the University of Massachusetts Medical School. Animals in the 17–21st generation of brother × sister matings, with an expected diabetes frequency of 40–80% were housed under specific pathogen-free (SPF) barrier conditions wherein they received laboratory chow and water ad libitum. All animals were tested for glycosuria three times weekly. Rats were considered diabetic if urine glucose was 2–4+ with TesTape and plasma glucose values (Beckman Instruments, Fullerton, CA) exceeded 200 mg/dl.

Antibodies. MRC OX6, OX8, OX17, OX19, W3/13, W3/25, OX35, OX38, and OX52 mAb were produced by hybridoma cell lines obtained from Drs. A. F. Williams and D. W. Mason, Oxford University (Oxford, United Kingdom) (21–23). 3JP producing cells were received from Dr. Charles Janeway, Yale University (New Haven, CT). The isotypes and reactivity of these mAbs are listed in Table I. The hybridoma cell lines were cultured under standard tissue culture conditions, and the undiluted mAbs containing TCS were stored at −20°C until the time for injection or use in the identification of lymphocyte cell type by flow cytometry. Although the mAb concentrations in the TCS were not quantified, each lot of TCS produced was tested by flow cytometry with normal rat spleen cells to insure the presence of saturating concentrations of the specific antibody. Fluoresceinated $F(ab')_2$ goat anti-mouse IgG (H and L chains), absorbed to remove crossreactivity with rat IgG, was obtained from Cappel Laboratories, Cochranville, PA.

Animal Injections and Disposition. Litters were randomized into groups and injected i.p. three times weekly with TCS containing the appropriate mAb, as follows: weeks 1 and 2, 1.0 ml i.p. per rat; weeks 3 and 4, 1.5 ml i.p. per rat; weeks 5 and 6, 2.0 ml i.p. per rat; weeks 7–14, 2.5 ml i.p. per rat. Controls received injections of tissue culture media (RPMI), or were uninjected. Representative nondiabetic animals were sacrificed after 5, 9, and 14 wk of injections for the analysis of splenic lymphocytes by flow cytometry. Diabetics were killed at detection of diabetes, and the remaining nondiabetics were killed at 18 wk, i.e., 4 wk after the end of injections.

Morphologic Studies. At autopsy, pancreatic, and thyroid tissues were fixed in Bouin's solution for routine light microscopic examination (by A. A. Like) of H and E–stained paraffin sections without knowledge of the treatment group or physiologic status of the animal. Pancreatic sections were reviewed for the presence of lymphocytic insulitis and end-stage islets, which are the characteristic islet lesions of diabetic BB rats. Lymphocytic insulitis is defined as the presence of lymphocytes surrounding and infiltrating the pancreatic islets (1–3). The term end-stage islet is defined as an irregular cluster of residual islet cells that persist after destruction of the insulin-synthesizing β cells. Thyroid sections were examined for evidence of lymphocytic thyroiditis, defined as the presence of lymphocytes infiltrating among and distorting the thyroid follicles (24).

Autoantibodies. Serum samples of untreated, OX8-, and OX19-injected animals were assayed for autoantibodies reacting with smooth muscle and thyroid colloid by indirect immunofluorescence (25).

Separation and Flow Cytometry of Splenic and Peripheral Blood Lymphocytes. Spleen cells were dispersed in cold RPMI tissue culture medium containing 5% newborn calf serum and 0.1% azide, and passed through Nitex filters. Splenic red blood cells were lysed with 0.15 M NH_4Cl in Tris buffer, pH 7.4. Peripheral blood leukocytes were separated by centrifugation of EDTA-treated whole blood in Wintrobe tubes, separation of the buffy coat, and lysing of red blood cells with NH_4Cl. After counting (Coulter Electronics, Inc., Hileah, FL), $3–5 \times 10^6$ cell aliquots were subjected to a primary incubation with the appropriate mAb in undiluted TCS. The secondary incubation used FITC-conjugated $F(ab')_2$ goat anti–mouse IgG (Cappell Laboratories) at a working concentration of 20 μg/ml. Normal rat serum (final concentration 10%) was added to reduce further the crossreactivity to rat IgG. The labeled cells were then examined in a FACS 440 Flow Cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). For each experiment, we analyzed 25,000 viable cells having the forward and 90° light scattering characteristics of lymphocytes. For two-color fluorescence analysis, 4×10^6 peripheral blood leucocytes of animals treated with OX8 or OX19 TCS for 10–14 wk were incubated with undiluted OX8 or OX52 TCS. The cells were then incubated with FITC–goat anti–mouse IgG, washed, and incubated with medium containing 200 μg/ml mouse IgG to block remaining active sites on the goat anti–mouse IgG. The cells were then incubated with biotinylated OX19 (26). After washing, the cells were incubated with 20 μl phycoerythrin-avidin (Becton Dickinson Immunocytometry Systems) diluted with 30 μl of tissue culture medium. The cells were then fixed with 0.5% paraformaldehyde and examined with a FACS 440 flow cytometer. Data from the two-color fluorescence experiments were displayed in contour plots.

Chromium-release Cytotoxicity Assay. Microcytotoxicity assays were carried out as described (27). YAC-1, a cell line extremely sensitive to lysis mediated by NK cells (28), was labelled with $Na^{51}Cr$ (Amersham Corp., Arlington Heights, IL) for use as target cells. Effector cells were splenic mononuclear cells. Three or more E/T ratios ranging from 200:1 to 3:1 were tested on microtiter plates with 10^4 target cells/well. Medium was added to target cells for spontaneous lysis determinations, and 1% NP-40 was added for determination of 100% lysis. Assays were incubated for 4–6 h in a humidified 37°C, 5% CO_2 in air incubator. Plates were centrifuged before harvest, and one-half of the supernatant fluid collected for radioactive counting in a Beckman gamma 5500 counter, (Beckman Instruments, Palo Alto, CA). The quadruplicate samples had standard deviations of <5% of the mean. Spontaneous release in different assays ranged from 5 to 12%.

TABLE II
Effects of mAb Injections on Splenic Lymphocyte Subsets

Injections	*n*	Percentage (mean ± SEM) of cells staining positively with:				
		OX19	W3/13	W3/25	OX8	OX6
None	12	7.3 ± 0.8	12.3 ± 1.3	7.2 ± 0.9	7.1 ± 0.8	59.2 ± 5.8
OX19	14	1.9 ± 0.3*	10.9 ± 1.6	5.2 ± 0.6‡	6.8 ± 1.3	56.0 ± 6.6
OX8	13	8.5 ± 0.7	14.6 ± 2.9	9.2 ± 0.7	1.3 ± 0.2§	61.9 ± 5.2
W3/25	4	6.8 ± 1.0	22.5 ± 5.8	9.0 ± 1.3	9.3 ± 2.4	67.5 ± 9.2
OX35	6	7.3 ± 1.5	10.4 ± 1.5	8.7 ± 1.6	6.8 ± 1.0	50.1 ± 6.9
OX38	7	7.9 ± 0.5	12.1 ± 1.4	8.6 ± 0.6	6.7 ± 0.9	65.7 ± 4.6
OX6	6	7.6 ± 0.9	8.7 ± 1.1	8.3 ± 1.1	7.0 ± 0.4	72.7 ± 4.0

The data were derived from aglycosuric animals sacrificed after 5, 9, and 14 wk of TCS injections. The splenic lymphocyte subsets were similar at each time point, and were therefore pooled.

* $p < 0.05$ for OX19 treatment vs. all other groups. SNK and LSD with BFA. $p < 0.001$ for OX19 vs. all other groups (except W3/25). LSD with BFA.

‡ $p < 0.05$ for W3/25 injection vs. OX8 treatment. SNK and LSD with BFA.

§ $p < 0.05$ for OX8 treatment vs. all other groups. SNK and LSD with BFA. $p < 0.01$ for OX8 treatment vs. no injection and OX19 injection LSD with BFA.

Percent specific ^{51}Cr release = 100 × [(cpm test sample) − (cpm medium control)]/[(cpm NP-40 control) − (cpm medium control)].

Statistical Procedures. One-way analyses of variance were used to compare the effects of the various treatment regimens on splenic and peripheral lymphocyte subsets. Individual pairs of groups were compared using a Student-Newman-Keuls (SNK) as well as a Fisher's least significant difference (LSD) Test with a Bonferonni adjustment (BFA) to compensate for additive type I errors. The effects of treatment on the frequency of diabetes, insulitis, and thyroiditis were analyzed using Fisher's exact test with BFA. Cytotoxicity data were analyzed using a student's *t* test. The effects of OX8 and OX19 treatment on autoantibody frequency were compared with the Fisher's exact test.

Results

Effects of Antibody Injections on Lymphocyte Subsets. Splenic lymphocyte subsets of uninjected diabetes-prone rats revealed the anticipated profound T lymphopenia and reduced percentages of W3/25 and OX8 subsets (Table II) (26, 29–31). OX6-staining B lymphocytes were reciprocally increased. Injections of TCS containing OX19 mAb significantly reduced the mean percentage of $OX19^+$ splenic lymphocytes. OX19-injected animals, however, did not reveal significant reductions of $W3/13^+$ and $W3/25^+$ cells, raising the possibility that OX19 mAb injections may have resulted in clearance of splenic T lymphocytes as well as modulation of OX19 membrane antigens from surviving splenic T cells. Injections of TCS containing OX8 mAb selectively and significantly reduced the percentage of $OX8^+$ splenic lymphocytes, without a detectable effect on other subsets. None of the TCS containing mAb against the helper/inducer T cells and Ia^+ and B cell subsets showed measurable effects on splenic $W3/25^+$ or other lymphocyte subsets (Table II). Similarly, the RPMI-injected rats and animals injected with 3JP mAb revealed no alterations in spleen cell subsets (data not shown).

To evaluate the possibility that OX19 TCS injections resulted in both a reduction of T cell numbers as well as the modulation of specific antigens from the target cell surface membranes, two-color flow cytometry analyses were

TABLE III
Two-color Flow Cytometry of Peripheral Blood Lymphocyte Subsets

Injections	*n*	Percentage (mean ± SEM) of cells staining with:			
		$OX8^+/OX19^-$ (NK)	$OX8^-/OX19^+$ (helper/inducer)	$OX8^+/OX19^+$ (cytotoxic/ suppressor)	$OX52^+/OX19^+$ (Pan-T)
None	7	5.3 ± 1.1	6.2 ± 0.77	0.51 ± 0.06*	6.4 ± 0.82
OX19	12	9.03 ± 1.2	1.08 ± 0.44‡	0.16 ± 0.043	0.82 ± 0.34§
OX8	9	0.56 ± 0.4‖	7.79 ± 1.1	0.21 ± 0.04	ND

* $p < 0.05$ for untreated vs. OX19- or OX8-treated rats. SNK. $p < 0.01$ for untreated vs. OX19- or OX8-treated rats. LSD with BFA.
‡ $p < 0.05$ for OX19 vs. untreated and OX8 treated rats. SNK. $p < 0.001$ for OX19 vs. untreated and OX8-treated rats. LSD with BFA.
§ $p < 0.05$ for OX19-injected vs. untreated rats. SNK. $p < 0.001$ for OX19-injected vs. untreated rats. LSD with BFA.
‖ $p < 0.05$ for OX8-treated vs. untreated and OX19-treated rats. SNK and LSD with BFA.

performed to compare the frequency of PBL staining with the noncompeting pan–T cell mAbs OX52 and OX19. OX52 and OX19 react with distinct proteins on the cell surface membranes of all rat peripheral T cells (21, 23). The data contained in Table III and illustrated in Fig. 1 *B* indicate that all PBL of untreated diabetes-prone rats stained after incubation with OX52 were also stained after a secondary incubation with OX19. Table III and Fig. 1, *C* and *D* indicate that PBL of OX19-treated animals revealed equivalent (virtually complete) reductions in the numbers of $OX19^+$ and $OX52^+$ cells. These data suggest that modulation of OX19 membrane antigen was not responsible for the reduced frequency of $OX19^+$ cells. Were antigen modulation a factor, one would expect disparate numbers of residual $OX52^+$ and $OX19^+$ cells.

Since peripheral T cells ($OX19^+$) are comprised of nonoverlapping helper/inducer ($W3/25^+$) and cytotoxic/suppressor ($OX8^+$) subsets, the $OX8^-$, $OX19^+$ cells are equivalent to the helper/inducer subset (21, 26). Table III and Fig. 1 *A* confirm the virtual absence of classical thymus-derived cytotoxic/suppressor ($OX8^+$, $OX19^+$) cells among untreated diabetes-prone BB/Wor rats (26). Table III and Fig. 1, *A* and *C* reveal that OX19 injections significantly and profoundly reduced the frequency of circulating $OX8^-$, $OX19^+$ (helper) cells, but did not significantly alter $OX8^+$ subsets. In contrast, OX8 treatment resulted in a significant reduction of NK ($OX8^+$, $OX19^-$) cells. The frequency of helper ($OX8^-$, $OX19^+$) cells was not significantly altered by OX8 treatments (Fig. 1 *E* and Table III).

Effects of Antibody Treatment on Disease. Table IV illustrates the frequency of diabetes among the several treatment groups. Injections of TCS containing either OX19 or OX8 profoundly reduced the incidence of diabetes from 61 to 0 and 12%, respectively ($p < 0.001$). Treatment with TCS containing OX17, W3/25, or OX35 significantly reduced the frequency of diabetes when compared with uninjected controls, but marginally when compared with animals injected with tissue culture media. When the Fisher's exact test stand-alone p values of the several treatment groups were corrected for the likelihood of spurious results with a BFA, only the OX8- and OX19-injected animals evidenced significant

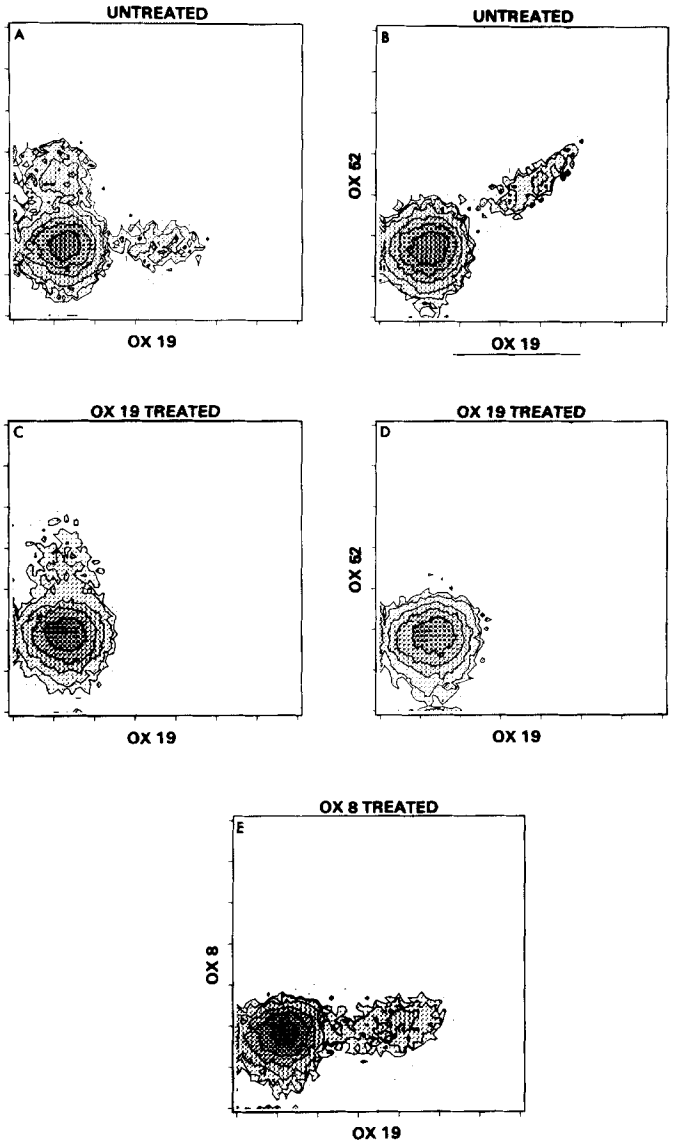

FIGURE 1. Contour plots of PBL stained with OX8 or OX52 followed by FITC–goat anti–mouse IgG, biotinylated OX19 and phycoerythrin-avidin. (*A*) PBL from untreated rat reveal 9.9% $OX8^+$, $OX19^-$, 5.5% $OX8^-$, $OX19^+$, and 0% $OX8^+$, $OX19^+$. (*B*) PBL from untreated rat show 0.4% $OX52^+$, $OX19^-$, 0.6% $OX52^-$, $OX19^+$, and 5.6% $OX52^+$, $OX19^+$. (*C*) PBL from OX19-treated rat reveal 8.7% $OX8^+$, $OX19^-$, 0.6% $OX8^-$, $OX19^+$, and 0.1% $OX8^+$, $OX19^+$. (*D*) PBL from OX19-treated rat show 1.8% $OX52^+$, $OX19^-$, 0.8% $OX52^-$, $OX19^+$, and 0% $OX52^+$, $OX19^+$. (*E*) PBL from OX8-treated rat show 0.3% $OX8^+$, $OX19^-$, 13.1% $OX8^-$, $OX19^+$, and 0.3% $OX8^+$, $OX19^+$.

protection against diabetes ($p < 0.01$) (Table IV). Injections with OX38, OX6, or 3JP did not protect against diabetes, even when the data were statistically examined without BFA.

Another parameter of protection was revealed when the pancreatic islets of the autopsied rats were examined histologically. The diabetic members of each

TABLE IV
Frequency of Diabetes after mAb Injections

Injections	Incidence of diabetes	Significance*
None	43/70 (61)‡	NS
RPMI	17/27 (63)	NS
OX19	0/49 (0)	$p < 0.001$§‖
OX8	11/90 (12)	$p < 0.001$§‖
W3/25	12/35 (34)	$p < 0.02$§
		$p < 0.05$‖
OX35	14/36 (39)	$p < 0.05$§
OX38	11/20 (55)	NS
OX6	28/56 (50)	NS
3JP	10/16 (63)	NS
OX17	7/22 (32)	$p < 0.03$§
		$p < 0.05$‖

The data included in Tables IV and V are the combined results of eight separate experiments. 3 of 8 experiments included RPMI-injected rats as controls, and 5 of 8 experiments included uninjected controls. After correction of stand-alone p values with BFA, only OX8- and OX19-injected rats were protected against diabetes ($p < 0.01$).

* Fischer's exact test.

‡ Percent in parentheses.

§ Compared with uninjected control.

‖ Compared with RPMI-injected control.

TABLE V
Frequency of Diabetes, Insulitis and Thyroiditis

Injections	Diabetes	Insulitis*	Thyroiditis
None	43/70 (61)	10/18 (45)	11/18 (61)
RPMI	17/27 (63)	ND	ND
OX19	0/49 (0)	0/43 (0)‡	2/34 (6)‡
OX8	11/90 (12)	44/57 (77)	26/42 (62)
W3/25	12/35 (34)	12/15 (80)	10/14 (71)
OX35	14/36 (39)	13/22 (59)	8/23 (35)
OX38	11/20 (55)	8/9 (89)	ND
OX6	28/56 (50)	3/10 (30)	6/12 (50)

Numbers in parentheses represent percent incidence.

* Frequency of insulitis and thyroiditis in the absence of hyperglycemia.

‡ $p < 0.001$ OX19 treatment vs. all other groups.

treatment group could not be differentiated: all revealed marked insulitis with extensive loss of β cells and end-stage islets. Histologically, the islets of the nondiabetic rats were not uniform. None of the 43 normoglycemic OX19-treated rats, and 44 of 57 (77%) normoglycemic OX8-treated rats revealed lymphocytic insulitis (Table V). The frequency of insulitis among nondiabetic animals of each of the other treatment groups tended to be greater than the incidence of diabetes. The numbers of animals in these groups were relatively small, and the frequencies of insulitis were not significantly different. A third measure of protection became evident when the incidence of lymphocytic thyroiditis was evaluated among nondiabetic rats. Only the OX19 treatment group was protected against thyroiditis (Table V).

TABLE VI
Serum Autoantibodies after mAb Injections

Injections	Autoantibodies*	Intensity of fluorescence‡
None	6/10 (60)	2.4 ± 0.5
OX8	12/13 (92)§,‖	2.9 ± 0.2
OX19	1/11 (9)¶	1.5

* An individual serum sample was considered autoantibody-positive if it reacted with either or both thyroid colloid and smooth muscle.
‡ Intensity of fluorescence of each sample was evaluated semiquantitatively (1–4+). Mean intensity (±SEM) was calculated from the positive sera.
§ $p < 0.01$ for OX8 treatment vs. no injections.
‖ $p < 0.001$ for OX8 treatment vs. OX19.
¶ $p < 0.03$ for OX19 treatment vs. no injections.

The incidence of diabetes, insulitis, and thyroiditis were the same among animals killed after 14 wk, the last week of TCS injections, and after 18 wk, 4 wk after the end of mAb treatment (data not shown). A final parameter of immunologic intervention was demonstrated when the sera of untreated, OX8- and OX19-injected animals were examined for anti–thyroid colloid and –smooth muscle antibodies. While OX19 TCS reduced the frequency of autoantibody-positive sera from 60 to 9% ($p < 0.03$), OX8 injections appeared to increase both the frequency and intensity of autoantibodies (Table VI).

OX8 Depletion of NK Cells. The absence of the $OX19^+$, $OX8^+$ T cytotoxic/suppressor cell population in diabetes-prone BB/Wor rats, and the fact that virtually all $OX8^+$ cells in these animals are NK cells (26, 32) led us to examine the effects of OX8 and OX19 TCS injections on functional NK activity. Splenocytes of untreated normoglycemic DB-PRN animals and nondiabetic rats treated with OX8 or OX19 TCS for 10–14 wk were tested against NK-sensitive YAC-1 target cells in a ^{51}Cr-release assay. Splenic mononuclear leukocytes isolated from untreated DB-PRN rats had high NK lytic activity, and mediated 30–50% killing of YAC-1 target cells at E/T ratios of 200:1 (Fig. 2, DB-PRN). Splenic mononuclear leukocytes isolated from OX8-treated animals were dramatically reduced in NK lytic activity and mediated only 10–15% lysis of YAC-1 target cells under similar conditions (Fig. 2, OX8-treated DB-PRN). DB-RES W-line animals are inherently low in the proportion of NK cells and NK cell function (33), and NK cell activity mediated by splenic cells isolated from the OX8-treated DB-PRN animals was comparable to that mediated by cells isolated from the DB-RES rats (Fig. 2). Under conditions wherein splenic effector cells isolated from pooled and individual untreated DB-PRN rats in six different experiments had a geometric mean lysis of 29%, splenic cells isolated from OX8-treated rats had a geometric mean lysis of 11%. The difference between the two groups was highly significant ($p < 0.001$). In contrast, injections of OX19 TCS had no significant effect on NK cell activity (Fig. 3).

Discussion

The results reported above indicate that chronic injections of TCS containing mAb that bind to all rat T cells ($OX19^+$) or to cytotoxic/suppressor and NK cells

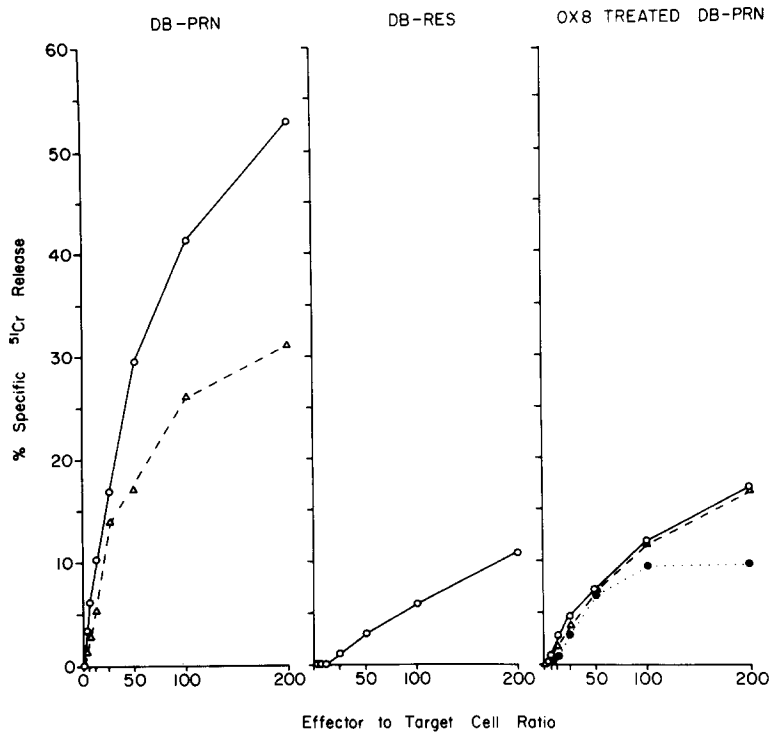

FIGURE 2. Titration of NK cell activity. Mononuclear leukocytes were isolated from spleens of untreated DB-PRN and DB-RES rats or DB-PRN animals injected with OX8 mAb for 14 wk. Spleen cells from each of two animals were pooled. Samples were taken for flow cytometry and cytolytic activity. Killing was measured in a 5-h ^{51}Cr-release assay against YAC-1 target cells. The percent specific ^{51}Cr release at different E/T ratios is shown. The percentage of $OX8^+$, $OX19^-$ cells for each preparation was as follows. DB-PRN: 6.3 (○), 3.6 (△); DB-RES: 3.1 (○); OX8-treated DB-PRN: 1.0 (○), 0.2 (△), 0.2 (●).

($OX8^+$) will achieve stable reductions of the respective lymphocyte subsets and provide profound (OX8) or complete (OX19) protection against BB/Wor diabetes. Although it is unambiguous that OX8 injections selectively reduced only the frequency of $OX8^+$ cells (i.e., NK cells) in diabetes-prone BB/Wor rats, the results of OX19 injections require further analysis. The splenic lymphocytes of OX19-treated animals studied with the technique of single-color flow cytometry revealed (Table II) markedly reduced numbers of $OX19^+$ cells, but only marginal reductions of helper ($W3/25^+$) and $W3/13^+$ cells. In contrast, PBL of a subsequent group of OX19-treated animals studied by two-color flow cytometry revealed profound reductions of both $OX52^+$, $OX19^+$ (pan-T) and $OX8^-$, $OX19^+$ (helper) cells. Since the absolute number and staining intensity of $W3/25^+$ helper/inducer lymphocytes are markedly reduced in DB-PRN animals, these cells are technically difficult to quantify with accuracy using single-color flow cytometry. We have observed that this subset of T cells is more accurately counted by two-color flow cytometry after sequential incubation with OX8 and OX19, both of which result in staining of greater intensity. Furthermore, the interpretation of data concerning $W3/13^+$ and $W3/25^+$ cells derived from spleen cell suspensions may be complicated by the presence of plasma cells and NK cells

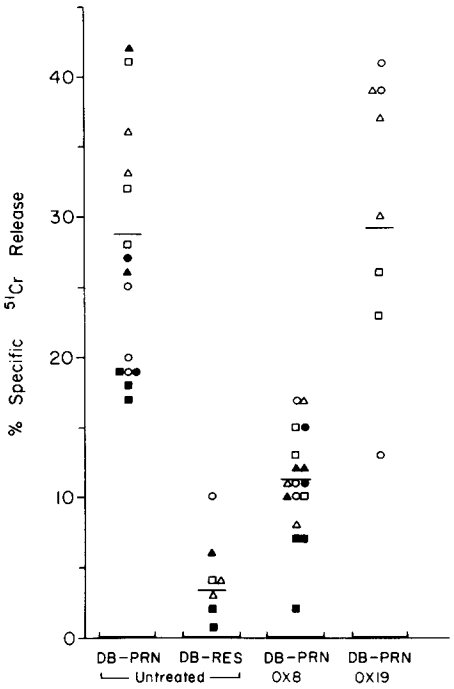

FIGURE 3. Specificity of OX8 treatments for depletion of NK cell activity. NK cell activity mediated by spleen cells isolated from untreated DP-PRN, DP-RES, OX8-treated DP-PRN, and OX19-treated DP-PRN rats was examined in 5–7-h ^{51}Cr-release assays against YAC-1 target cells. Results shown were obtained with cells isolated from individual animals in five different experiments (○, ●, □, ■, △) and cells pooled from two animals in one experiment (▲). The E/T ratio was the same for all samples tested on a given day, and were as follows: ○, 50:1; ●, 50:1; □, 33:1; ■, 100:1; △, 50:1; ▲, 100:1. Bars represent geometric means. The statistical significances of the differences between experimental groups, and untreated DB-PRN were: DB-RES $p < 0.001$; OX8-treated DB-PRN $p < 0.001$; OX19-treated DP-PRN, $p > 0.5$.

that are W3/13$^+$ (21), and by the presence of macrophages, which express W3/25 antigens (22). The technique of two-color flow cytometry using peripheral blood leukocytes is therefore a more precise method of delineating T cell subsets in DB-PRN rats. We are therefore convinced that the reduced numbers of helper/inducer cells in OX19-treated rats illustrated in Fig. 1 and Table III are correct, and that OX19 treatment of DB-PRN rats in fact reduced the number of W3/25$^+$ T lymphocytes.

The results reported above also indicate that injections of OX8 profoundly protected and injections of OX19 TCS completely protected against diabetes. Furthermore, although a substantial number of nondiabetic OX8-treated animals showed signs of lymphocytic insulitis, this was not observed after OX19 injections. We interpret these results to indicate a role for both NK cells and helper/inducer cells in the pathogenesis of BB diabetes.

The evidence that OX8 TCS injections reduced OX8$^+$ cell numbers is supported by the in vitro NK assays. The reduction of phenotypic NK cells (OX8$^+$, OX19$^-$ by flow cytometry) (Table III) correlated with a significant reduction in the lysis of YAC-1 cells in vitro (Figs. 2 and 3).

The evidence indicating that NK cells contribute to the induction of diabetes in the DB-PRN rat is now very persuasive. Both NK cell numbers and activity are elevated on a per-cell basis in untreated DB-PRN as compared to DP-RES rats (Fig. 2) (33). Data reported previously (19) indicate that $OX8^+$ cells are localized at the site of the insulitis lesion in the BB/Wor rat. The data presented here indicate that injections of OX8 mAb prevented diabetes and also resulted in the clearing of $OX8^+$ cells from the spleen (Table II) and blood (Table III). Since virtually all of the $OX8^+$ cells in DB-PRN rats belong to the NK cell subset (26), and since OX8 injections both prevented diabetes and depleted NK cell activity (Figs. 2 and 3), we believe that NK cells contribute to the mechanism of β cell destruction. Although we can not exclude the possibility that another $OX8^+$, $OX19^-$ subset is (also) involved, this does not appear likely, since all $OX8^+$ cells in the DP-PRN rat are also brightly stained with a second NK cell marker, AGM1 (33).

Although OX19 injections protected against diabetes, they did not reduce the number of NK cells or NK cell–mediated lysis of YAC-1 cells. Therefore NK cells cannot be solely responsible for BB/Wor diabetes. It is reasonable therefore to suggest that the helper/inducer cell subset is required for continued (lymphokine) stimulation of NK cell activity and/or the stimulation of B lymphocyte synthesis of islet cell–surface antibodies, which may precede the temporal onset of diabetes in the BB rat (8). Indirect support for the latter possibility is derived from the evidence that autoantibodies directed against smooth muscle and thyroid colloid were significantly reduced in OX19-injected rats (Table IV).

An alternate explanation for the reduction of autoantibody synthesis in OX19-treated rats is suggested by the reported (34) presence of a small subset of B lymphocytes that bear the Ly-1 phenotype and are capable of synthesizing autoantibodies in normal and immunodeficient mice. OX19 is thought to be the analog of murine Ly-1 in the rat. To date, no such $OX19^+$ B cell subset has been described. If, however, a comparable $OX19^+$ B cell subset exists in the immunodeficient BB/Wor rat, it is possible that the protective effects of OX19 treatment could be due in part to a direct effect on these B lymphocytes.

Injections of TCS containing mAb that bind selectively to helper/inducer cells (W3/25, OX35, and OX38) and to cells with class II (Ia) surface membrane antigens (OX6, 3JP, and OX17) did not reduce the percentages of helper or Ia^+ splenic cell subsets. This was noted even after injections of mAb of the IgG2a class (OX35, OX38, and 3JP). The results reported above indicate that marginal reductions in the frequency of diabetes were realized in W3/25- and OX35 TCS–treated animals without reduced helper cell numbers. Furthermore, normoglycemic recipients of anti–helper/inducer cell mAb did not reveal reduced frequencies of insulitis. It is not known whether prolonged injections of larger quantities of mAb would have reduced the number of $W3/25^+$ cells and protected more profoundly against diabetes (and insulitis). Injections of 6 mg of W3/25 ascites mAb to each of 10 diabetes-prone rats over a 3-wk interval neither reduced splenic $W3/25^+$ cells nor prevented diabetes (data not illustrated).

Brostoff and Mason (35) have reported the reduction in duration and amelioration of severity of experimental allergic encephalomyelitis (EAE) in Lewis rats after injections of milligram quantities of W3/25 without associated reductions

of circulating T helper cells. Those workers speculated that the injected mAb might impair helper cell function without cell loss. In confirmation of Brostoff's observations was the report that injections of anti–helper cell (L3T4) mAb prevented and ameliorated EAE in mice (36). Anti-L3T4 mAb also reduced the severity of the autoimmune syndrome in NZB/NZW F_1 mice (37). In both groups of mouse experiments, mAb treatment reduced circulating L3T4 cells.

The marginal protection against diabetes that followed injections of OX17 (IaE) but not OX6 or 3JP (IaA) confirms the published report of Boitard, et al. (38). Those investigators reported that injections of purified anti-IaE mAb provided marginal protection against BB/Wor diabetes and lymphocytic thyroiditis, when considered individually. Protection, however, reached statistical significance only when the frequency of diabetes and thyroiditis were combined. Boitard et al. did not report the effects of their treatments on lymphocyte subsets.

Finally, we note with interest that there was an unanticipated relationship between the IgG class of the injected mAb and the reduction of T cell subsets. Hence, OX8 and OX19, both IgG1 isotypes, induced profound reductions of PBL and splenocyte subsets, and protected against diabetes. OX35, OX38, and 3JP are all IgG2a isotypes, and in the quantities injected did not reduce the number of their respective subsets. This contrasts with the report of Cobbold et al. (39), wherein IgG2B but not IgG1 mAb was responsible for in vivo helper T cell elimination in mice.

Summary

Diabetes-prone BioBreeding/Worcester (BB/Wor) rats received thrice weekly injections of mAb against antigens expressed on the surface of all T cells (OX19), cytotoxic/suppressor, and NK cells (OX8), helper/inducer cells (W3/25, OX35, OX38), and Ia^+ cells (OX6, 3JP, OX17). Treatment with OX8 or OX19 achieved stable reductions of splenic and peripheral blood NK cells and helper/inducer T lymphocytes, respectively, and protected against diabetes. OX19 injections also prevented lymphocytic insulitis, thyroiditis, and the synthesis of autoantibodies to thyroid colloid and smooth muscle antigens. OX8 injections reduced splenic NK-mediated YAC-1 cell lysis, but did not prevent insulitis, thyroiditis, or autoantibody synthesis. Injections of mAb specific for antigens on the surface of helper/inducer cells, and for cells expressing IaE antigens provided marginal protection against diabetes without reductions of phenotypic subsets. These findings suggest that pancreatic β cell destruction in the spontaneously diabetic BB/Wor rat is mediated by the combined action of NK and helper/inducer cells.

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