

Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice

(autoimmunity/diabetes)

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Communicated by N. Le Douarin, August 23, 1993

ABSTRACT Anti-CD3 monoclonal antibodies suppress immune responses by transient T-cell depletion and antigenic modulation of the CD3/T-cell receptor complex. Anti-CD3 treatment of adult nonobese diabetic (NOD) mice, a spontaneous model of T-cell-mediated autoimmune insulin-dependent diabetes mellitus, significantly inhibits the autoimmune process. Short-term low-dose anti-CD3 treatment (5 $\mu\text{g}/\text{day}$ i.v. for 5 consecutive days) prevented the occurrence of an accelerated form of the disease induced by cyclophosphamide. More unexpectedly, when applied to adult NOD females within 7 days of the onset of full-blown diabetes, the same anti-CD3 regimen induced a complete remission of overt disease (i.e., a return to permanent normoglycemia) in 64–80% of mice. This remission was durable (>4 months) and was not associated with the disappearance of insulinitis (mononuclear cell infiltration of the islets). The immunosuppression was apparently specific for β -cell-associated antigens, since mice showing anti-CD3-induced remission rejected histoincompatible skin grafts normally, whereas they did not destroy syngeneic islet grafts, unlike control untreated overtly diabetic NOD females. These results open major therapeutic perspectives. They strongly suggest that self-tolerance can be restored in adult mice once autoimmunity is fully established and confirm that this effect can be obtained by transient targeting of the CD3/T-cell receptor without massive T-cell debulking.

Autoimmune insulin-dependent diabetes mellitus (IDDM) in nonobese diabetic (NOD) mice ensues from progressive loss of self-tolerance to β -cell-associated antigens (1, 2). Immunointervention using monoclonal antibodies (mAbs) targeting one component of the antigen recognition triad constituted by the antigen itself, major histocompatibility complex molecules, and the CD3/T-cell receptor (TCR) complex (and its coreceptors), when applied early in the ontogeny [i.e., in neonates (3, 4) or before the onset of clinical manifestations], has been shown to prevent spontaneous or experimentally induced IDDM [by cell transfer or cyclophosphamide (CY)] (5–9). mAbs so far successfully tested include anti-major histocompatibility complex class II (9), anti-TCR $\alpha\beta$ (8), anti-CD3 (3), and anti-CD4 and anti-CD8 (1, 2, 5, 6).

In contrast, treatment of NOD mice with established autoimmunity and full-blown diabetes due to irreversible destruction of a large proportion of the insulin-secreting β -cell mass remains a major challenge. Apart from its obvious clinical interest, the possibility of restoring self-tolerance in overtly autoimmune animals has fundamental implications that are of the utmost importance for our understanding of the mechanisms regulating autoimmune responses.

It has been extensively shown in models of tolerance induction to alloantigens in adult animals that active mechanisms participating in the control of the alloimmune re-

sponse, afforded by cell-mediated anergy and infectious tolerance, can be triggered by distinct T-cell-directed therapies (10–12).

Our aim was thus to determine whether adult NOD mice are still sensitive to T-cell-directed immunointervention once the autoimmune process has led to significant target tissue damage.

MATERIALS AND METHODS

Mice and Antibodies. NOD (K^d , I-A^{NOD}, Db) and C57BL/6 (H-2^b) mice were bred in our animal facilities under specific pathogen-free conditions. NOD mice were monitored for glycosuria and fasting glycemia with colorimetric strips (Glukotest and Haemoglukotest-Reflolux F; Boehringer Mannheim). Diabetes was defined as permanent fasting glycemia >4 g/liter.

The hybridoma producing 145 2C11 (hamster IgG, antimurine CD3) was kindly provided by J. A. Bluestone (Chicago University) (13). The purified endotoxin-free antibody used for *in vivo* treatment was produced by Celltech (Berkshire, U.K.). Anti-CD3 F(ab')₂ was prepared by conventional pepsin digestion of the entire antibody molecule [2-hr digestion at 37°C in pH 3 buffer, pepsin at a 2% (wt/vol) final concentration]. Digested F(ab')₂ fragments were purified using a Sepharose CL-4B-protein A (Pharmacia) affinity chromatography column followed by an Ultrogel AcA54 column (Pharmacia).

mAb Treatment. (i) Ten-week-old NOD females presenting spontaneous insulinitis were treated i.v. with 5 μg of anti-CD3 on 5 consecutive days. Controls received normal hamster immunoglobulins. Pancreata were collected at various times, and serial sections were stained with hematoxylin/eosin to score mononuclear cell infiltration.

(ii) Nondiabetic 8-week-old NOD males and females received two CY (Endoxan-Asta, Laboratoires Lucie, Colombes, France) injections [200 mg/kg (1 mg/ml in saline) i.p.] 14 days apart. In CY-treated mice anti-CD3 treatment was started 1 day prior to the second CY injection (day 14) and administered for 5 consecutive days at a low dose (5 $\mu\text{g}/\text{day}$ i.v. from day 13 to day 17). Anti-CD3 F(ab')₂ fragments were administered for an identical period but at higher doses (10 or 50 $\mu\text{g}/\text{day}$ i.v. from day 13 to day 17). Controls received normal hamster immunoglobulins. The animals were tested for the appearance of diabetes.

(iii) Mice showing stable glycosuria and fasting glycemia >4 g/liter on two consecutive occasions were considered diabetic and randomized to receive either anti-CD3 (5 $\mu\text{g}/\text{day}$ for 5 consecutive days) or normal hamster immunoglobulins. Complete remission was defined as the disappearance of glycosuria and a return to normal glycemia.

The distribution of the main T-cell subsets was monitored using the T-cell-specific mAbs CD3 (145 2C11), TCR $\alpha\beta$ (H57 597), CD4 (GK1.5), CD8 (YTS 169), and CD116/CD18 (Mac1). Fluorescent cells were scored using a flow cytometer (FACScan, Becton Dickinson).

Skin and Islet Grafting. Pellets of 500–700 islets, isolated from male NOD pancreata using conventional collagenase digestion and Ficoll gradient separation (14), were transplanted under the recipient's kidney capsule. Islet survival was evaluated by blood glucose measurement.

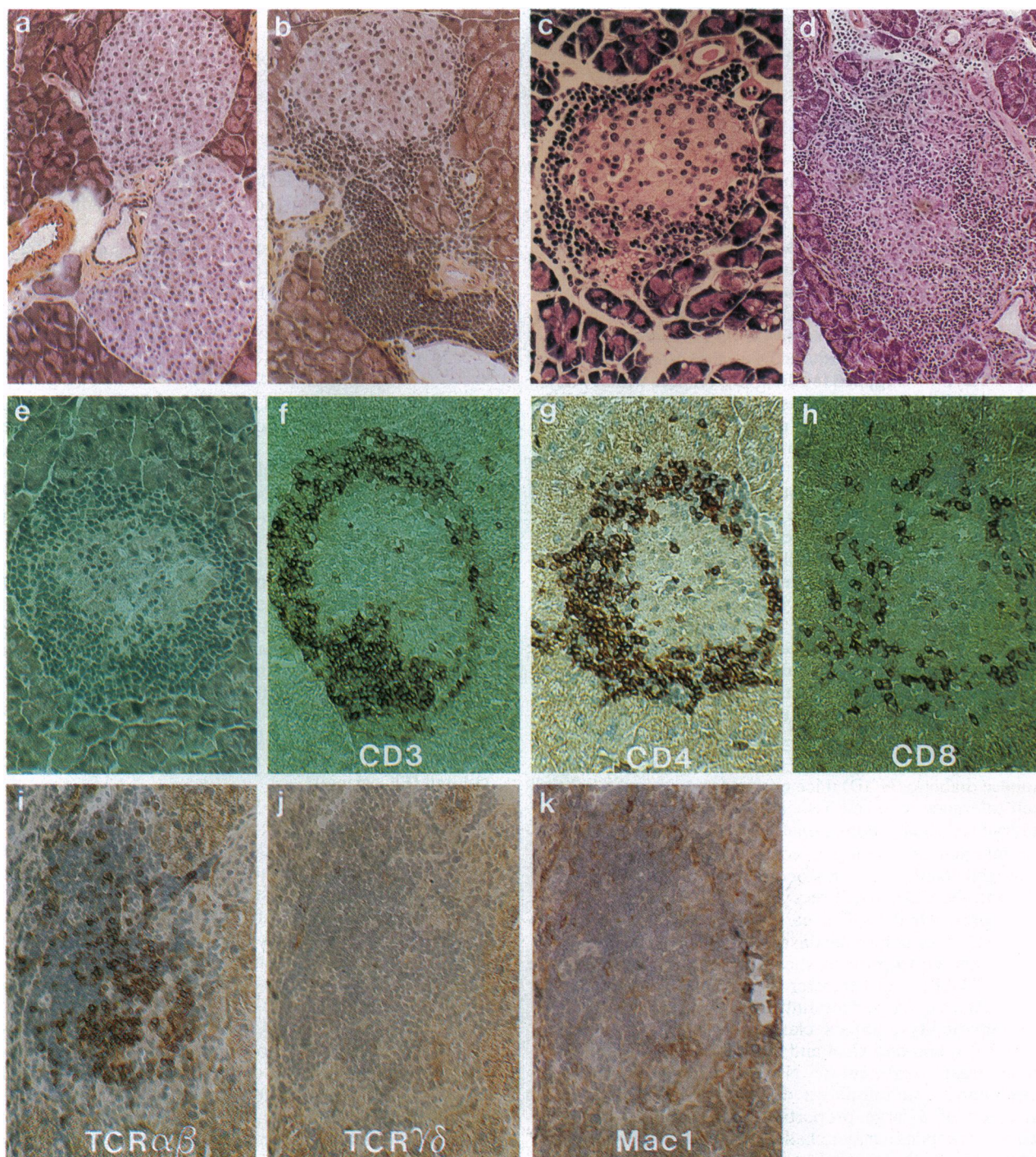


FIG. 1. Histological and immunohistochemical analysis of insulinitis in mice showing anti-CD3-induced remission. (a–d) Progressive development of spontaneous full-blown insulinitis in untreated NOD mice (hematoxylin/eosin staining). According to our histological scores of infiltration, the microphotographs show: normal islets (grade 0; a), focal or peripheral insulitis (no destruction of endocrine cells) (grade 1; b and c), and invasive destructive insulinitis (d). (e–k) Insulinitis pattern observed in mice showing anti-CD3-induced remission. Immunostaining of distinct cell subsets (f–k) was performed using mAbs against CD3 (145 2C11), TCR $\alpha\beta$ (H57 597), TCR $\gamma\delta$, CD4 (GK 1.5), CD8 (YTS 169) (15), and Mac1. In euglycemic mice ($n = 14$) 10–20 weeks after anti-CD3 treatment, a mean of 40% infiltrated islets was observed. Of these, the vast majority [$34\% \pm 16.6\%$ (mean \pm SD) out of the 40%] presented focal or peripheral (grade 1) insulinitis (e shows a representative islet). Panels f–h correspond to this same islet stained with different mAbs; the infiltrate included CD3⁺ (f), CD4⁺ (g), and CD8⁺ (h) cells. (i–k) A second islet. The microphotographs show that CD3⁺ infiltrating cells are TCR $\alpha\beta$ ⁺ (i); TCR $\gamma\delta$ cells were almost absent (j); very few Mac1⁺ cells were detected.

C57BL/6 mice were used as skin donors. Tail skin grafts (0.5 cm × 0.5 cm) were transplanted onto the lateral thoracic wall of the recipient and then covered with gauze for the first few days. Graft survival was documented daily starting on the fourth day.

Statistical Analysis. Results were analyzed using the χ^2 test or the nonparametric Mann and Whitney U test, as appropriate.

RESULTS

Effect of Anti-CD3 Treatment on Spontaneous Insulinitis and CY-Induced Diabetes. Before treatment, 37% of islets in NOD females showed infiltration [mean \pm SEM: 25.3% \pm 4.1% grade 1 and 11.7% \pm 6.2% grade 2 (Fig. 1) insulinitis] (Fig. 2a). A significant fall in the proportion of islets showing grade 1 insulinitis (22% \pm 6.4% vs. 47% \pm 2.9% in controls; $P < 0.001$)

and grade 2 insulinitis (2% \pm 2% vs. 13.7% \pm 2.3% in controls; $P < 0.001$) was observed at the end of anti-CD3 treatment (day 6), but reinvasion started by day 10 (Fig. 2a).

As shown on Fig. 2 b-d, a 5-day treatment with low-dose anti-CD3 (5 μ g/day i.v.) or anti-CD3 F(ab')₂ (50 μ g/day i.v.) starting 1 day prior to the second CY injection (days 13-17) reproducibly prevented or reversed CY-induced diabetes (Fig. 2 b-d).

Anti-CD3-Induced Remission of Established Diabetes in NOD Females. Overt diabetes in NOD females was identified by twice-weekly screening for glycosuria; when glycosuric, mice were tested for hyperglycemia. Mice showing fasting glycemia > 4 g/liter on two consecutive occasions were randomized (to avoid sampling bias) to receive short-term low-dose treatment with either anti-CD3 (5 μ g/day for 5 consecutive days) or normal hamster immunoglobulins. Progressive remission of established disease—namely, disap-

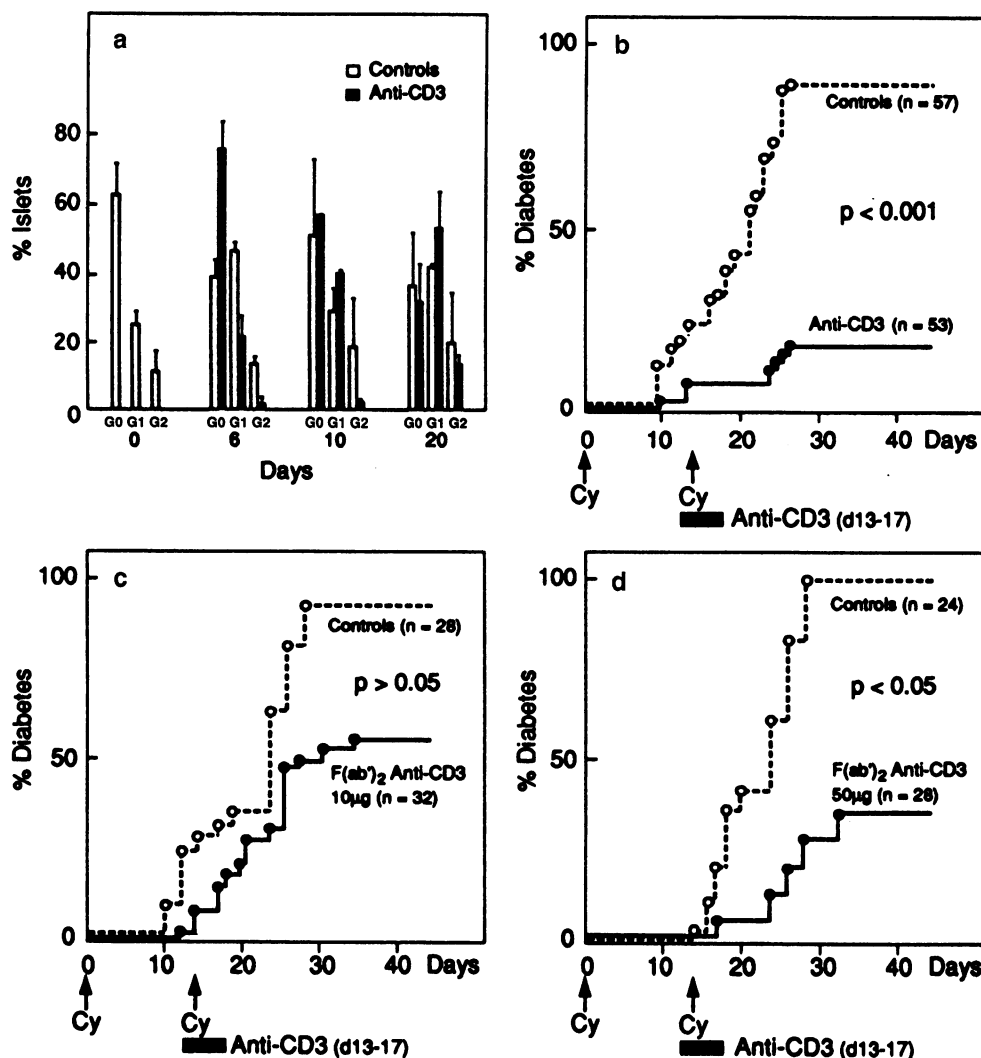


FIG. 2. Low-dose anti-CD3 treatment of spontaneous insulinitis and CY-induced diabetes. (a) Effect of anti-CD3 treatment on spontaneous insulinitis. A significant fall in the proportion of islets showing grade 1 (G1) or grade 2 (G2) insulinitis was observed at the end of anti-CD3 treatment (day 6), but gradual reinvasion started by day 10. G0, grade 0 (normal islets). Spleen T-cell subsets were monitored in NOD mice treated with anti-CD3; results are expressed as percentages. Day 0: CD3⁺, 78.4 \pm 2.7; CD4⁺, 52.6 \pm 2.2; CD8⁺, 24 \pm 1.5. Day 13: CD3⁺, 40.2 \pm 1.8; CD4⁺, 33.7 \pm 4.5; CD8⁺, 30.1 \pm 1.4; deduced percentages of CD3⁻ CD4⁺ or CD8⁺ antigenically modulated cells, 23.6. Day 30: CD3⁺, 65.3 \pm 2.6; CD4⁺, 48.3 \pm 3.8; CD8⁺, 16.6 \pm 2.5; absence of CD3⁻ antigenically modulated cells. (b) Effect of anti-CD3 treatment on CY-induced IDDM. Results represent cumulated data from five different experiments. They were analyzed using the χ^2 test. (c and d) Effect of anti-CD3 F(ab')₂ treatment on CY-induced IDDM. Results represent cumulated data from three (c) and two (d) different experiments. They were analyzed using the χ^2 test. Spleen T-cell subsets were monitored in NOD mice treated with anti-CD3 F(ab')₂ fragments; results are expressed as percentages. Day 0: CD3⁺, 82.1 \pm 2.2; CD4⁺, 52.4 \pm 3.3; CD8⁺, 27.5 \pm 1.7. Day 13: CD3⁺, 52.5 \pm 2.6; CD4⁺, 39.1 \pm 2.7; CD8⁺, 28.3 \pm 1.9; deduced percentage of CD3⁻ CD4⁺ or CD8⁺ antigenically modulated cells, 14.9%. Day 20: CD3⁺, 84.3 \pm 1.6; CD4⁺, 52.4 \pm 2.0; CD8⁺, 27.8 \pm 1.8; absence of CD3⁻ antigenically modulated cells.

pearance of glycosuria and a return to normal glycemia—was noted in 64–80% of anti-CD3-treated mice within 2–4 weeks of the last mAb injection (Fig. 3). Interestingly, focal or peripheral insulinitis was observed in mice showing anti-CD3-induced remission (Fig. 1 *e-k*).

Syngeneic Islet Graft Survival in NOD Mice Showing Anti-CD3-Induced Remission. Fresh syngeneic islets were transplanted under the kidney capsule of mice still overtly diabetic 6–10 weeks after the end of anti-CD3 treatment (as shown in Fig. 3, 20–36% of anti-CD3-treated NOD mice did not enter metabolic remission) as well as control mice (overtly diabetic NOD females not treated with anti-CD3). Syngeneic islets transplanted into the controls were destroyed within 2–4 days but survived well in anti-CD3-treated animals and led to full metabolic reconstitution (Fig. 4).

Allogeneic Skin Graft Rejection in NOD Mice Showing Anti-CD3-Induced Remission. Allogeneic skin graft survival was identical in mice showing stable anti-CD3-induced remission (9–19 weeks after the end of anti-CD3 treatment) and in age-matched controls (Table 1).

DISCUSSION

Anti-CD3 mAbs are potent immunosuppressive agents that essentially act by reversibly clearing CD3/TCR complexes from the T-cell membrane by antigenic modulation (16, 17). Short-term low-dose anti-CD3 treatment thus precludes the prolonged T-cell depletion observed with polyclonal anti-lymphocyte antibodies and some anti-CD4 or anti-CD8 mAbs (5, 10).

We first determined the dose of anti-CD3 that is well tolerated by adult NOD mice. In fact, in adult mice, the first anti-CD3 injection causes an acute physical syndrome linked to a monocyte-dependent T-cell activation leading to massive cytokine release (18, 19). A dose of 5 μg per injection was selected.

We then assessed the capacity of this dose given for 5 consecutive days to influence the course of established

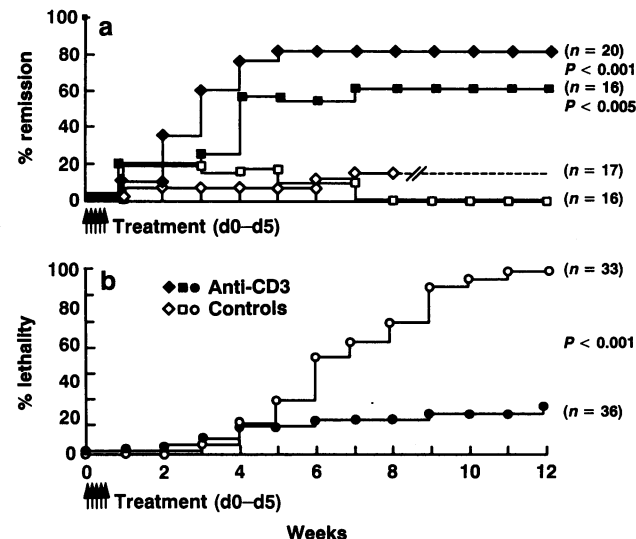


FIG. 3. Anti-CD3-induced remission of established diabetes in NOD females. Overtly diabetic NOD mice (stable fasting glycemia >4 g/liter) were randomized to receive either anti-CD3 (5 μg /day for 5 consecutive days) or normal hamster immunoglobulins. Complete remission was defined as the disappearance of glycosuria and a return to normal glycemia. Results were analyzed using the χ^2 test. Actuarial curves of remission (a) and mortality (b) in anti-CD3-treated and control mice are shown. The data in a correspond to two independent experiments in which 37 and 32 mice were randomized (90 and 110 mice were initially screened for diabetes). The data in b represent the cumulated data from the two experiments.

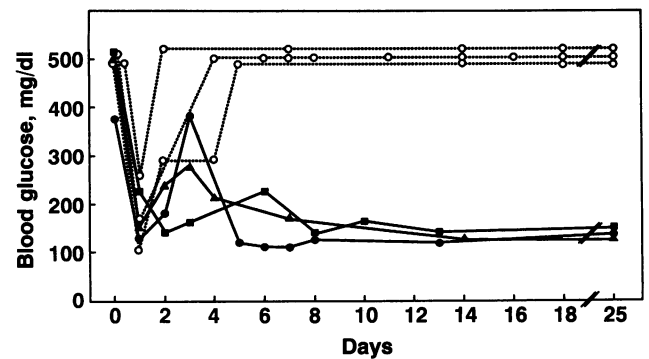


FIG. 4. Survival of syngeneic islet transplants in mice showing anti-CD3-induced remission. Fresh NOD islets were transplanted under the kidney capsule of mice still overtly diabetic 6–10 weeks after the end of anti-CD3 treatment (solid curves). These islets showed prolonged survival and allowed full metabolic reconstitution; the follow-up of three representative animals is presented. In control animals (untreated overtly diabetic NOD females; dotted curves), syngeneic islets were rapidly destroyed.

spontaneous insulinitis in NOD mice. Spontaneous diabetes in our breeding colony appears at 15–28 weeks of age in 50–60% of females and is preceded by insulinitis, which develops by 5–8 weeks. A 5-day course of anti-CD3 (5 μg /day) in 10-week-old NOD females induced a significant reduction in insulinitis. However, this effect was transient: within 2 weeks of the last injection, the insulinitis pattern was identical in anti-CD3-treated and control animals.

We then examined the effect of anti-CD3 on the course of an accelerated model of IDDM induced by CY (20, 21). Anti-CD3 treatment started 1 day prior to the second CY injection—when ≈ 15 –20% of mice are already diabetic—was highly effective both in preventing progression to IDDM and in reversing overt disease. F(ab')₂ fragments of anti-CD3 mAb, as already reported in other models (22), showed similar immunosuppressive activity but only if given at higher doses.

These data extend to an autoimmune model our previous observation that this short-term low-dose anti-CD3 treatment is highly effective in prolonging the survival of a fully mismatched skin allograft (23).

The same protocol was used to determine whether anti-CD3 could stop the progression of spontaneous ongoing autoimmune IDDM. Overtly diabetic mice were randomized to receive either anti-CD3 or irrelevant hamster immunoglobulins. As reflected by the disappearance of glycosuria and a return to normal glycemia, diabetes regressed in 64% and 80% of the anti-CD3-treated mice in two independent experiments. This remission was significantly more frequent than the rare, transient remission observed in control NOD mice treated with irrelevant hamster immunoglobulins. The anti-CD3-induced remissions presented a number of unexpected features: (i) they developed over 2–4 weeks, suggesting the participation of active phenomena, (ii) the effect was durable (until sacrifice at >4 months), (iii) remission was

Table 1. Skin allograft rejection in anti-CD3-treated and control mice

| Mice | Mean \pm SD survival time, days |
|-------------------|-----------------------------------|
| Anti-CD3 treated* | 10.2 \pm 1.8 |
| Controls† | 10.4 \pm 1.2 |

*NOD females showing IDDM remission 8–19 weeks after the end of anti-CD3 treatment received a C57BL/6 skin transplant ($n = 6$).

†Age-matched NOD females, not treated with anti-CD3, received a C57BL/6 skin transplant ($n = 6$).

associated with only partial and transient T-cell depletion (CD3⁺ cell counts returned to normal 15–20 days after the end of treatment), and (iv) clinical remission was maintained despite the presence of peripheral (but not invasive/destructive) insulinitis including CD3⁺ TCR $\alpha\beta^+$ CD4⁺ CD8⁺ cells, assessed 10–20 weeks after anti-CD3 treatment. Importantly, mice showing anti-CD3-induced remission were not globally immunocompromised since they had a normal capacity to reject skin allografts. In contrast, anti-CD3-treated animals did not destroy syngeneic islet grafts, further stressing the specificity of the anti-CD3-induced remission for β -cell-associated antigens. Recurrence of the autoimmune disease is usually observed within a few days when syngeneic islets grafts are implanted into untreated overtly diabetic NOD mice. The survival of syngeneic islets, which implies anti-CD3-mediated arrest of the autoimmune process, was independent of metabolic status. Indeed, syngeneic islets led to full metabolic reconstitution of NOD mice that were still diabetic 4 to 5 weeks after anti-CD3 treatment. This points to an essential pitfall of this model of established diabetes when metabolic reconstitution (reversal of hyperglycemic diabetic disease that is directly correlated to the available insulin-secreting β -cell mass) is taken as the only index of autoimmune status. Thus, mice showing anti-CD3-mediated arrest of the autoimmune process may still be hyperglycemic if the β -cell mass left at the time of treatment is insufficient to guarantee metabolic reconstitution (i.e., a return to normoglycemia).

Similar data have recently been reported by Maki *et al.* (24), who used polyclonal anti-lymphocyte serum or a combination of depleting anti-CD4 and anti-CD8 mAbs. However, at variance with our study, profound and prolonged T-cell depletion was required to achieve long-term remission of established diabetes. In addition, in Maki's study the diabetic mice also received insulin, which may have contributed to the preservation of remnant β cells, since insulin therapy can promote remission in human IDDM (25). It is also worth noting that treatment with high doses of an anti-TCR mAb achieved only transient remission of established diabetes in NOD females (8).

To our knowledge, our results provide the first indication that a short course of anti-CD3 given alone can restore self-tolerance in adult mice with established autoimmune diabetes. The effect is obtained at a very advanced stage of the disease process when, on the basis of indirect evidence, >70% of β cells have been destroyed.

The mechanism leading to the establishment of such long-term remission is unclear. Although direct testing for tolerance would imply the use of the triggering autoantigen(s), the nature of which is elusive despite the interesting candidates recently proposed (1, 2), the results of syngeneic islet and allogeneic skin transplants strongly argue for the specificity of the anti-CD3-induced unresponsiveness. Extrapolating from other experimental situations (10–12), it is tempting to speculate that T-cell anergy or active T-cell-mediated suppressor circuits are active in our model. It is also possible that anti-CD3-induced T-cell activation participates in this effect.

Hayward and Shreiber (3) obtained permanent protection from diabetes by injecting NOD neonates with large, single doses of anti-CD3. However, at that age the antibody probably had a direct effect on either intrathymic T-cell education or on T-cell selection in the periphery. Neonatal anti-CD3 treatment totally prevented the occurrence of insulinitis (3),

whereas in our adult model anti-CD3-induced remission did not correlate with the disappearance of insulinitis.

If confirmed in humans, our results will open a new era in the immunotherapy of autoimmune diabetes. The possibility of avoiding continuous therapy would eliminate the risks of toxicity and long-term overimmunosuppression associated with most immunointervention regimens so far proposed for IDDM.

The authors are indebted to Dr. Jeffrey Bluestone (University of Chicago) and Professor Herman Waldmann (Addenbrokes Hospital, Cambridge, U.K.) for providing the 145 2C11 and YTS 169 mAbs, Mrs. Sylvie Merite for skillful technical assistance, Mrs. Maryline Calise for animal care, Mr. David Young for English editing, and Mrs. Karine Steiner for secretarial assistance.

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