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Immunology

A T-cell dormant state in the autoimmune process of nonobese diabetic mice treated with complete Freund's adjuvant

(insulin-dependent diabetes mellitus/autoimmunity/adjuvants)

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ABSTRACT **Three experimental manipulations showed the suppressive effect of injection of complete Freund's adjuvant (CFA) in the diabetic state of nonobese diabetic (NOD) mice. (*i*) Diabetes was inhibited in young NOD mice injected with the adjuvant. (*ii*) Recurrence of diabetes in mice transplanted with pancreatic islets was also inhibited when the recipients were injected with CFA. (*iii*) Injection of spleen lymphocytes from diabetic mice into male NOD mice transferred the diabetic state, but this transfer was markedly suppressed when the recipients were treated with CFA. In all three cases the spleen cells from the normoglycemic mice treated with CFA induced diabetes when transferred into NOD male mice. CFA, therefore, induces a state of T-cell dormancy, in which the islets are no longer subject to an immune attack.**

The autoimmune diabetic process of the nonobese diabetic (NOD) strain of mice is easily suppressed by a variety of inflammatory and/or immunological stimuli (1–7). These stimuli have ranged from infections with viruses or intracellular pathogenic bacteria to injection of complete Freund's adjuvant (CFA) (6, 7). Perhaps the effect of these different stimuli is to release the acute cytokines—such as interleukin-1 (IL-1), interleukin-6, or tumor necrosis factor (TNF)—that, in some way, modulate the autoimmune state. Indeed, there are reports showing the ameliorating effect of repeated injections of TNF (8, 9) or IL-1 (9) on the diabetic state of NOD mice. Intrigued by these surprising findings, we now attempt to define the status of the T cells in CFA-treated NOD mice that do not exhibit diabetes.

MATERIALS AND METHODS

Mice. Male NOD mice were bred in our own colony under specific pathogen-free conditions or purchased from Taconic (Germantown, NY). Female NOD mice were bred in our own colony. After reaching 16 weeks of age female mice were tested weekly for diabetes by analysis of urine glucose with Chemstrips-UG (Boehringer Mannheim). Blood glucose levels of >300 mg/dl usually were confirmed on the day of sacrifice. Blood glucose levels were measured on a Beckman glucose analyzer or an Ames Glucometer II on blood collected in heparinized capillary tubes from the retroorbital area.

CFA. CFA (Bacto adjuvant H37-RA from Difco) was prepared by emulsification with an equal volume of phosphate-buffered saline.

Experimental Conditions. Three sets of experimental conditions were evaluated. In each the diabetic process was inhibited by CFA injections; subsequently, lymphocytes were harvested from the spleens and transferred i.v. into male nondiabetic NOD mice that had received 790 R (1 R = 0.258 mC/kg) from a ^{137}Cs source (Gammacell 40, Atomic Energy, Ottawa)].

(*i*) The first condition consisted of NOD female mice that received CFA at 7–9 weeks of age (50 μl i.p. and 100 μl s.c. on the base of the tail). (*ii*) The second condition consisted of diabetic NOD that received islet transplants from male, nondiabetic NOD mice, followed by CFA (either 200 μl i.p. or 50 μl i.p. and 100 μl s.c.). The pancreatic islets of Langerhans were isolated by the method of Lacy and Kostianovsky (10) from 8- to 10-week-old male NOD mice. Briefly, islets were isolated from NOD pancreas by collagenase digestion and centrifugation over a discontinuous Ficoll gradient, cultured for 7 days at 24°C or 37°C in CMRL medium (GIBCO) adjusted to 150 mM glucose and supplemented with glutamine, penicillin at 100 μg/ml, streptomycin at 50 μg/ml, and 5% fetal calf serum.

(*iii*) The third manipulation consisted of consecutive adoptive transfer: male NOD mice received the lymphocytes from diabetic NOD mice and a day later received 200 μl of CFA i.p. The spleens were removed from diabetic females, usually within a week after the diagnosis of diabetes. The spleen cells were harvested at a later time from the recipients and transferred to a second set of irradiated male NOD mice, untreated with CFA.

Diabetes was defined as a reading of 300 mg/dl of blood glucose for 2 consecutive weeks.

RESULTS

NOD Mice Treated with CFA Did Not Exhibit Diabetes but Contained Autoimmune Lymphocytes. Two groups of 7- to 9-week-old prediabetic female mice were treated with CFA or PBS. By 114 days after treatment all 10 of the PBS-treated mice and two of the 10 CFA-treated mice were diabetic. The remaining CFA-treated mice remained healthy and normoglycemic until sacrifice at 215 days after treatment. At sacrifice histological examination of the pancreases of CFA-treated mice, which were at this time >260 days old, revealed many insulin-bearing cells within the islets with a heavy periislet lymphocytic infiltrate (data not shown). However, the infiltrating lymphocytes did not invade the islets, and no evidence of ongoing islet destruction was seen. Spleen cells from these long-term normoglycemic CFA-treated mice transferred disease to healthy syngeneic recipients (Fig. 1).

NOD Mice Transplanted with Islets Were Protected by CFA But Also Contained Autoimmune Lymphocytes. Spontaneously diabetic female NOD mice were engrafted with 500 syngeneic islets in the renal subcapsular space. Generally, within 48 hr of transplant a state of normoglycemia was restored in the recipient mice. However, diabetes recurred after ≈10 days (Fig. 2 *A* and *C*). Administration of CFA i.p. and s.c. in tail base 24 hr after transplant prevented disease recurrence (Fig. 2 *B* and *D*); these CFA-treated mice re-

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Abbreviations: CFA, complete Freund's adjuvant; IL-1, interleukin-1; TNF, tumor necrosis factor; NOD, nonobese diabetic.

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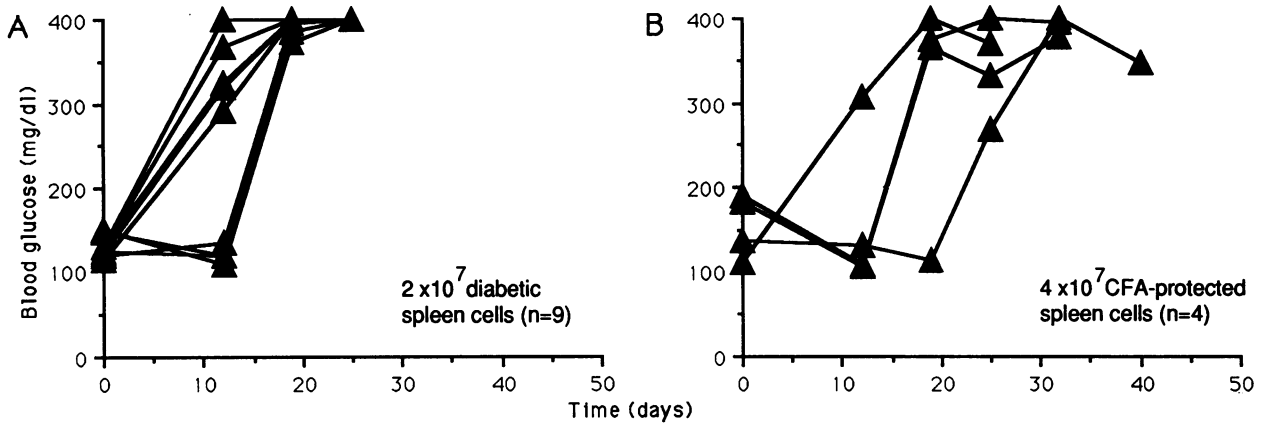

FIG. 1. Blood sugar levels in adoptive transfer recipients of spleen cells from diabetic (*A*) or CFA-protected (*B*) female mice. In *A*, mice were injected with 2×10^7 spleen cells from spontaneously diabetic female NOD mice. In *B*, recipient mice were injected with 4×10^7 spleen cells from four female NOD mice treated with CFA and killed after 215 days. Blood glucose of all four mice was <140 mg/dl 24 hr before sacrifice.

mained healthy (some were followed up to 176 days after transplant).

Histological examination of pancreas and engrafted kidneys of CFA-protected mice confirmed the absence of endogenous insulin-containing β cells in their pancreas and their presence under the kidney capsule (data not shown), thus confirming that the maintenance of normoglycemia was due to the engrafted islets rather than residual β cells in the pancreas. Adoptive transfer of spleen cells from recipients of islet graft and CFA-protected mice to healthy syngeneic mice resulted in disease transfer (Table 1).

Autoimmune Lymphocytes Transplanted into Nondiabetic NOD Mice Treated with CFA Did Not Transfer Diabetes. We also determined the status of T cells in adoptive transfer of disease from spontaneously diabetic mice to healthy syngeneic mice. We first confirmed that spleen cells from diabetic NOD mice adoptively transferred diabetes in healthy mice, depending on the presence of both $CD4^+$ and $CD8^+$ subsets of T cell (11–13). Over a 40-day period, CFA administered in a single i.p. dose 24 hr after spleen cell transfer prevented the development of diabetes in the recipient mice (Fig. 3 and Table 2); 94% of untreated and only 23% of CFA-treated recipients developed disease. We also found that the degree of protection varied inversely with the number of spleen cells transferred (data not shown). We found, too, that incomplete Freund's adjuvant gave some degree of protection. For

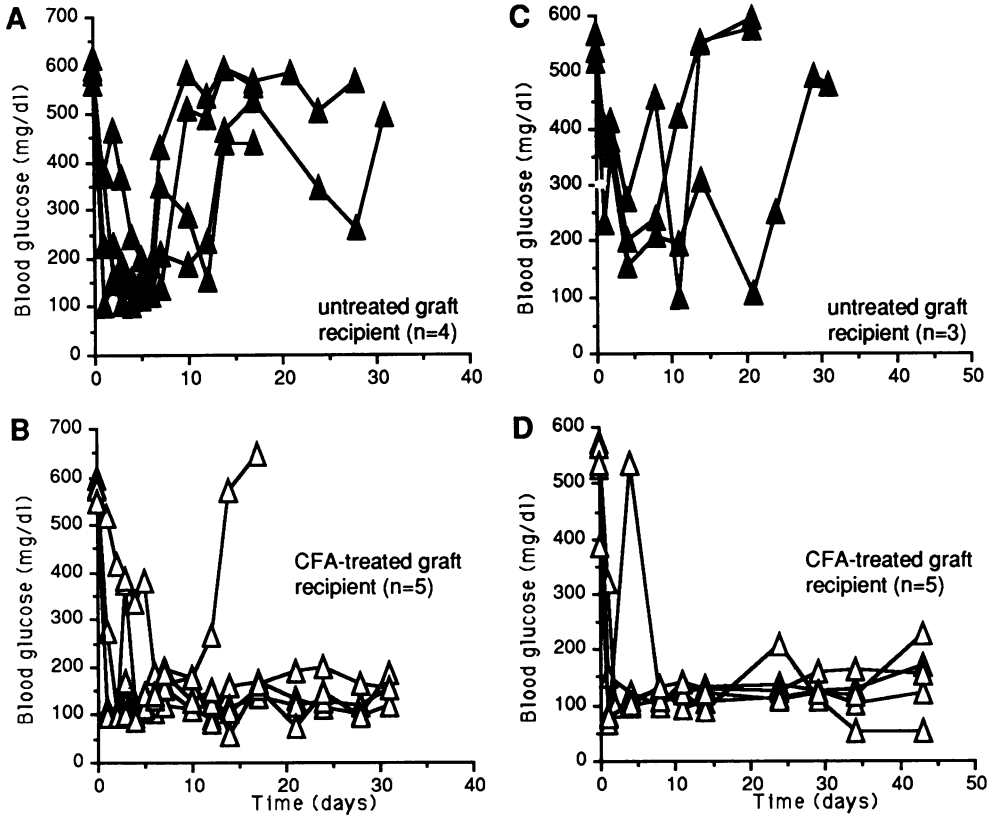

FIG. 2. Blood sugar levels in spontaneously diabetic mice engrafted with syngeneic islets and treated with CFA. Spontaneously diabetic female NOD mice were engrafted under the left kidney capsule with islets isolated from syngeneic male mice. Twenty-four hours after transplant the mice were either left untreated (*A* and *C*) or treated with CFA (*B* and *D*). Data are from two independent experiments. *A* and *B* represent one experiment; *C* and *D* represent a second experiment.

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Table 1. Spleen transfers from NOD mice receiving islet transplants

	Spleen treatment				
	+ CFA			− CFA	
Exp.	Interval, days	Cells, no. ($\times 10^7$)	Incidence, +/total	Cells, no. ($\times 10^7$)	Incidence, +/total
1	41	2	4/4	2*	4/4
1	55	1	0/3	1†	2/2
2	176	2	5/6	2†	9/9

Results of transfer of spleen cells into male NOD mice. Spleen cells were obtained from normoglycemic mice that received an islet transplant and were treated with CFA (+ CFA). Interval refers to the time between transplantation of islets and harvesting of spleen cells. The number of spleen cells are noted. Spleen cells were also obtained from diabetic mice that did not receive CFA (− CFA). Exp., experiment.

*Spleen donors were transplanted with islets but were not treated with CFA and then were maintained on insulin until the day of transfer (41 days later).

†Spleen donors were spontaneously diabetic mice that did not receive a transplant.

Table 2. Transfer of spleen cell into untreated or CFA-treated recipients

	Incidence of diabetes, diabetic mice/total mice	
Exp.	Untreated	CFA-treated
1	4/5	0/3
2	4/4	1/5
3	5/5	3/4
4	5/5	2/5
5	5/5	0/5
6	4/4	1/3
7	5/5	1/4
8	4/5	1/4
9	4/4	2/5
10	5/5	0/4
11	4/4	1/3
12	4/5	1/6
13	4/5	1/5
14	4/4	0/5
Incidence	61/65 (94%)	14/61 (23%)

This table summarizes all experiments involving transfer of diabetic spleen cells into either untreated or CFA-treated recipients. All mice were followed for 40 days.

example, 8 of 12 adoptive transfer recipients became diabetic after incomplete Freund's adjuvant treatment compared with 2 of 12 CFA-treated and 12 of 14 untreated mice. The ability of incomplete Freund's adjuvant to protect from disease transfer was not further investigated.

In another manipulation, CFA was administered to healthy male mice 3 days before spleen cell transfer. The ability of CFA to protect from disease transfer was drastically reduced (Fig. 4 *C* and *F*). Similar data, not shown, were obtained when CFA was administered 1 day before spleen cell transfer; three of five mice administered CFA on the day before transfer became diabetic by 18 days (the untreated and CFA-treated mice data from this experiment are shown in Fig. 3 *C* and *D*).

Spleen Cells from CFA-Treated Recipients of Autoimmune Lymphocytes Contained Disease-Causing T Cells. We performed serial adoptive transfers from diabetic mice into healthy syngeneic recipients. Spleen cells from diabetic mice were adoptively transferred to male NOD recipients. After 24

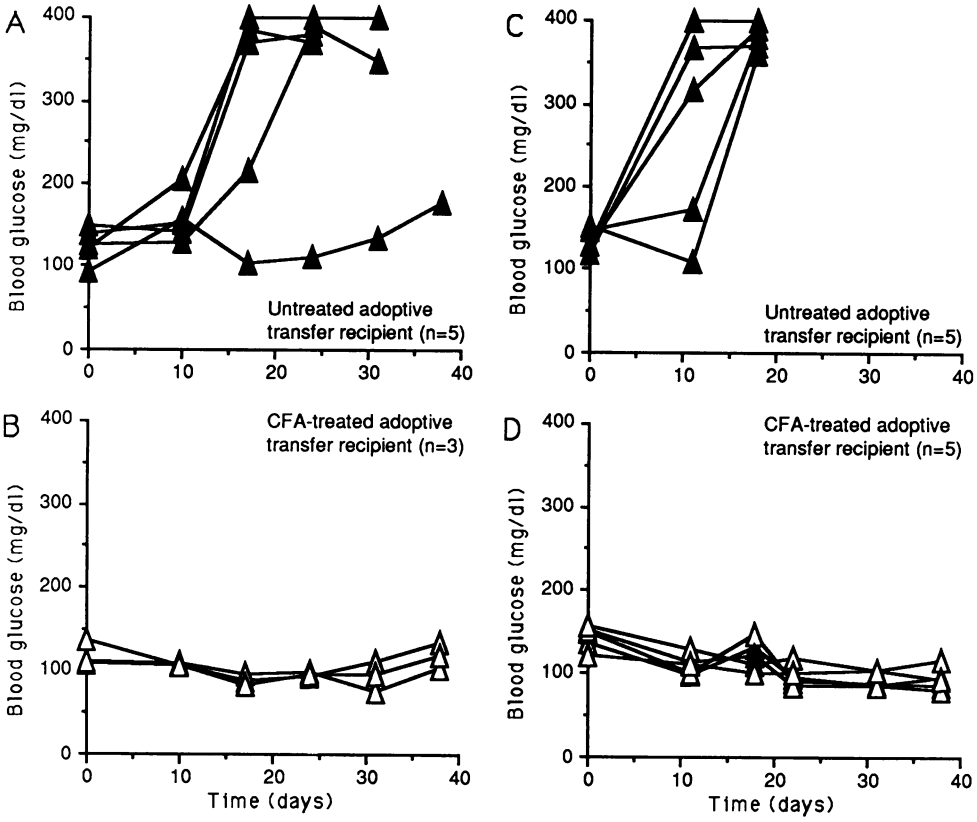

FIG. 3. Blood sugar levels in mice injected with diabetogenic T cells followed by CFA treatment. Eight- to 10-week-old male NOD mice were irradiated and injected with 3×10^7 spleen cells from spontaneously diabetic female mice. Twenty-four hours after adoptive transfer the recipient mice were either left untreated (*A* and *C*) or treated with CFA (*B* and *D*). Data are from two representative experiments. *A* and *B* represent one experiment; *C* and *D* represent a second experiment.

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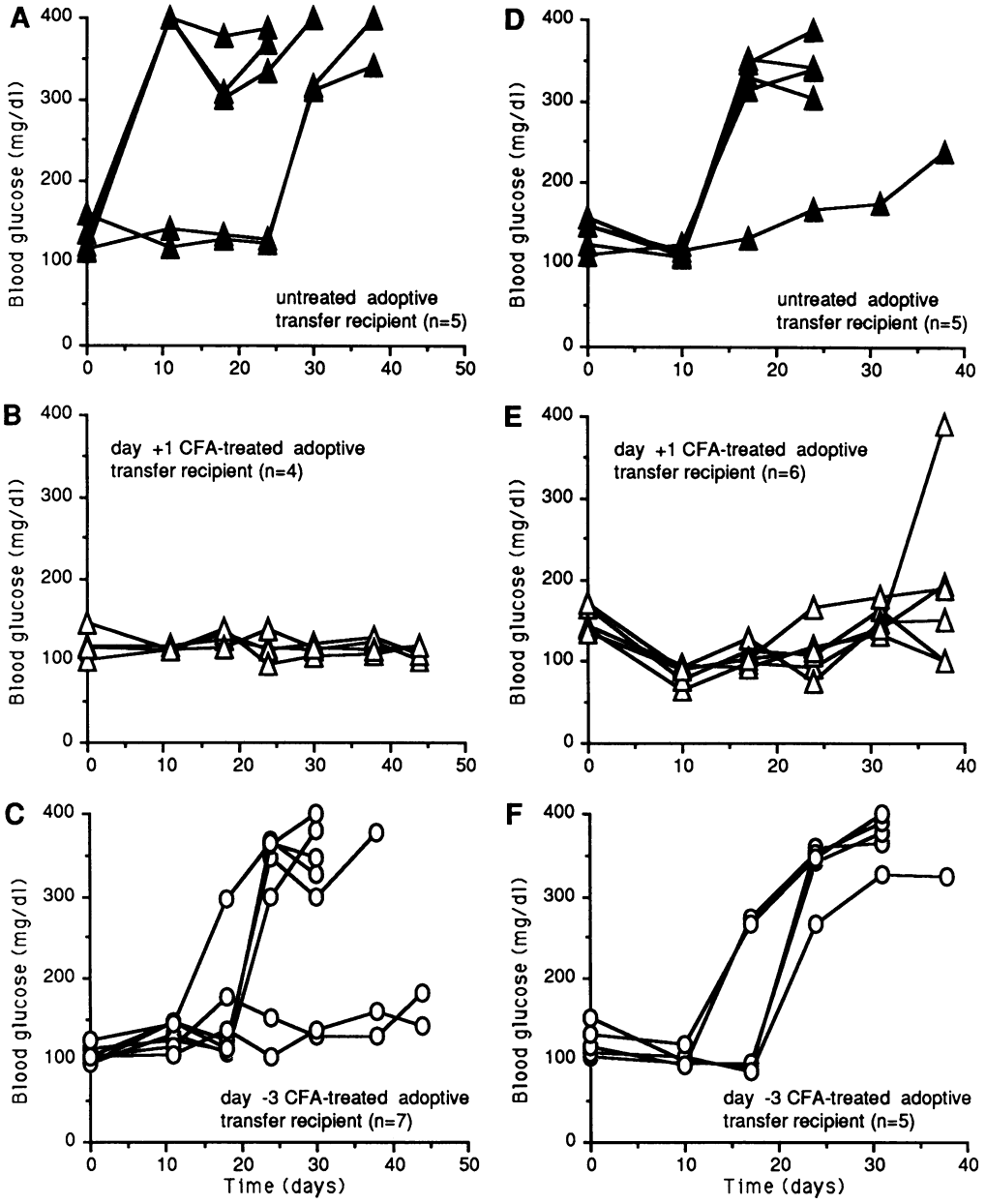

FIG. 4. Blood sugar levels in adoptive transfer recipients treated with CFA before inoculation with diabetogenic spleen cells. Healthy irradiated NOD males were injected i.v. with 2×10^7 spleen cells from spontaneously diabetic females. Recipient males were left untreated (*A* and *D*) or treated either with CFA 24 hr after adoptive transfer (*B* and *E*) or with CFA 72 hr before adoptive transfer (*C* and *F*). *A*, *B*, and *C* represent one experiment; *D*, *E*, and *F* represent a second experiment.

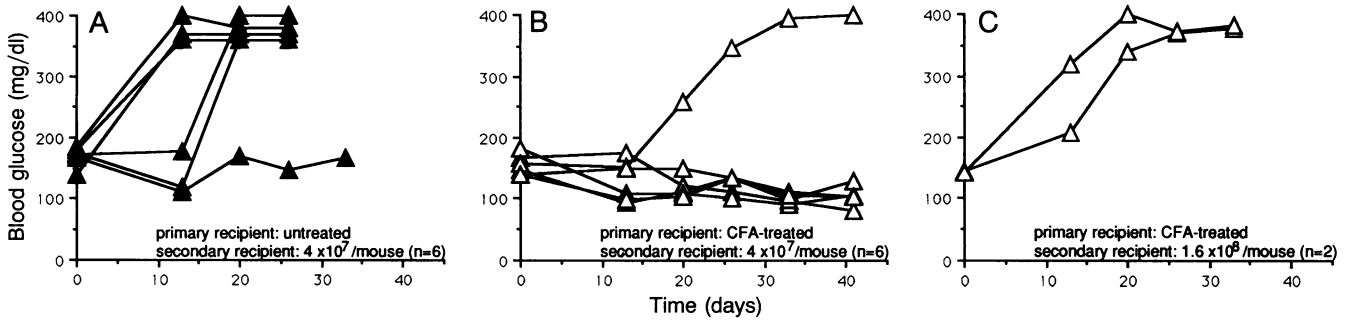

FIG. 5. Blood sugar levels in secondary recipients of spleen cells from primary adoptive transfer recipients treated or not treated with CFA. Healthy irradiated NOD males were injected i.v. with spleen cells from males injected 14 days previously with 3×10^7 spleen cells from spontaneously diabetic females either not treated (*A*) or treated with CFA (*B* and *C*). Blood sugars of all CFA-treated primary recipients used for secondary adoptive transfer were <160 mg/dl. Blood sugars of four of five untreated primary recipients used for secondary adoptive transfer were >300 mg/dl.

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hr 10 recipients were treated with CFA and five were left untreated. Fourteen days after transfer four of five untreated recipients had manifested blood glucose levels >300 mg/dl, while all CFA-treated mice remained normoglycemic. All untreated mice and five of the CFA-treated mice were sacrificed, and their spleen cells were adoptively transferred into a second round of healthy recipients. (The remaining five CFA-treated mice were maintained until day 39 after transfer, during which only one became diabetic.) The spleen cells from the untreated recipients transferred diabetes (five of six mice, Fig. 5*A*). The spleen cells from the CFA-treated mice were less efficient—one of four mice that received 4×10^7 cells became diabetic, but also two of the two mice that received a much higher inoculum (Fig. 5 *B* and *C*) became diabetic.

DISCUSSION

In agreement with previous investigations, we found that CFA protected NOD mice from the development of diabetes. Not only did CFA inhibit the development of the diabetic state, but it also inhibited diabetes when T cells were already activated, as noted in the transplant experiment (Fig. 2) and in the adoptive transfers (Fig. 3). Thus, the function of activated T cells to cause diabetes (which includes migration into the islets, recognition of their antigen, and killing of the β cells) could be profoundly inhibited, and for long periods of time, by the adjuvant. Notably, the T cells after CFA treatment were not tolerized but remained in the host, at face value, in a dormancy state. This dormant state was reactivated when such T cells were now transplanted into an irradiated recipient. Indeed, in three different conditions we could identify such T cells in the CFA-protected mice.

There are two striking issues in these experiments, which we can't explain but which deserve comment. (*i*) The periinsulitis characteristics of the early stage of the process was not ablated by CFA. The relationship between the periinsulitis and the invasive stage, where cells penetrate and kill the β cells, is unclear. Both stages could be part of the same process or distinctly different processes. (*ii*) The time between lymphocyte transfer and the CFA-injection effect had a time limit, a constraint clearly evident in the experiment of Fig. 4. Therefore, the CFA inhibition may not be caused by a widespread activation of cells in the various tissues. Most likely this inhibition concerns modulation of T cells, perhaps initiated by a burst of cytokines like IL-1 or TNF or others. Our interpretation is supported by the experimental results in which injection of TNF or IL-1 inhibited the transfer of diabetes (9).

It is surprising that CFA, a powerful adjuvant in common use for the enhancement of immune responses, should so strikingly inhibit the autoimmune process of NOD mice. The relevance of these findings to the general study of autoimmunity and immunity is as yet unknown. The most extensively studied model of autoimmune disease is experimental allergic encephalomyelitis in rodents. To induce experimental allergic encephalomyelitis susceptible animals must be immunized with autoantigen emulsified in CFA. However, reports indicate that CFA administered to rats before challenge with encephalitogenic antigen protects the animals from experimental allergic encephalomyelitis (14–16), and that muramyl dipeptide (17), a component of the mycobacteria of CFA, prevents the adoptive transfer of experimental allergic encephalomyelitis in guinea pigs. Thus, the phenomenon described in NOD probably operates in other T cell-mediated autoimmune diseases. Other experiments described in the literature also indicate the immunosuppressive capability of CFA (18–20).

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