Multiple low-dose streptozotocin-induced diabetes in the mouse. Evidence for stimulation of a cytotoxic cellular immune response against an insulin-producing beta cell line.

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Research Article

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Multiple Low-Dose Streptozotocin-Induced Diabetes in the Mouse

Evidence for Stimulation of a Cytotoxic Cellular Immune Response Against an Insulin-producing Beta Cell Line

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Abstract. Mice were examined for the presence of splenocytes specifically cytotoxic for a rat insulinoma cell line (RIN) during the induction of diabetes by streptozotocin (SZ) in multiple low doses (Multi-Strep). Cytotoxicity was quantitated by the release of ^{51}Cr from damaged cells. A low but statistically significant level of cytolysis (5%) by splenocytes was first detectable on day 8 after the first dose of SZ. The cytotoxicity reached a maximum of ~9% on day 10 and slowly decreased thereafter, becoming undetectable 42 d after SZ was first given. The time course of the in vitro cytotoxic response correlated with the degree of insulitis demonstrable in the pancreata of the Multi-Strep mice. The degree of cytotoxicity after Multi-Strep was related to the number of effector splenocytes to which the target RIN cells were exposed and was comparable to that detectable after immunization by intraperitoneal injection of RIN cells in normal mice. The cytotoxicity was specific for insulin-producing cells; syngeneic, allogeneic, and xenogeneic lymphocytes and lymphoblasts, 3T3 cells, and a human keratinocyte cell line were not specifically lysed by the splenocytes of the Multi-Strep mice. This phenomenon was limited to the Multi-Strep mice. Splenocytes from mice made diabetic by a single, high dose of SZ exhibited a very low level of cytotoxicity against the RIN cells. The cytotoxic response was also quantitated in splenocytes from control and Multi-Strep mice (10 d after the first dose of SZ) before and after culture with mitomycin-treated RIN cells in the presence of T cell growth factor (TCGF). The cytotoxicity of the Multi-Strep splenocytes was enhanced more than fivefold after such culture, suggesting the proliferation of an effector cell that could be stimulated and supported in vitro by TCGF. These results support the hypothesis that cell-mediated anti–beta cell autoimmunity may play a role in the destruction of the beta cells in this animal model. The stimulation of this response by TCGF may provide a tool by which enough cytotoxic effector cells could be obtained to establish their possible direct pathogenetic role in the induction of insulin-dependent diabetes. In addition, such cells will be a valuable tool to define the specific beta-cell antigens that may direct the highly selective cell-mediated destruction of these cells in experimental models and, perhaps, in human insulin-dependent diabetes mellitus.

Introduction

Several lines of evidence have been developed in recent years to support the hypothesis of an involvement of the immune system in the pathogenesis of insulin-dependent diabetes mellitus in humans as well as in certain animal models. The involvement of the immune system in the pathogenesis of diabetes induced in mice by multiple subdiabetogenic doses of the pancreatic beta-cell toxin, streptozotocin (SZ)[1], (1, 2) is somewhat controversial. The principal findings in the early

1. *Abbreviations used in this paper:* BAS, basal release; CMF, calcium-magnesium free-Hank's balanced salt solution; ConA, concanavalin A; CT, control; E/T, effector/target; EXP, experimental; ga, gauge; MAX, maximum release; TCGF, T cell growth factor; RIN, rat insulinoma cell line; SZ, streptozotocin.

reports on this Multi-Strep model included a delayed onset of hyperglycemia, the development of insulitis (3), and the possible activation of a C-type virus in the pancreatic beta cells (2, 4). These original observations were made in CD-1 mice. Subsequent studies, however, have revealed that inbred strains of mice vary considerably in the susceptibility to diabetes induced by Multi-Strep (5–9). Furthermore, a different susceptibility to Multi-Strep diabetes between male and female mice has been consistently demonstrated. Even in the most highly susceptible strains, only male mice develop hyperglycemia (8, 10, 11).

An immune component in the pathogenesis of diabetes in the Multi-Strep model was supported in an early study in which injection of anti–mouse lymphocyte serum partially (2) or completely (12) prevented diabetes in CD-1 mice. Buschard and Rygaard (13) subsequently reported that athymic nude mice did not develop diabetes after Multi-Strep whereas genetically similar mice with intact cellular immune responses were normally susceptible. Attempts to confirm this observation have not been consistent (14, 15), and further studies attempting to passively transfer the diabetes to nude mice have also been contradictory (10, 11, 16, 17). Buschard and Rygaard (13) reported that splenic splenocytes from Multi-Strep diabetic mice could transfer the diabetes to either syngeneic normal mice or nude mice. The degree of hyperglycemia induced, however, was mild and others have been unable to confirm this observation (14, 18, 19), although insulitis in some of the recipients was noted in the absence of significant hyperglycemia (18, 19).

A number of other immunological manipulations have been performed in an attempt to establish an immune component in Multi-Strep diabetes. The production of immune deficiency by irradiation prevented the development of diabetes after Multi-Strep. The susceptibility was restored by the partial reconstruction of immunity with a T lymphocyte-enriched preparation of splenocytes from syngeneic mice (17). A B lymphocyte-enriched preparation did not restore susceptibility. On the contrary, in C57BL/KsJ mice, immune deficiency induced by either thymectomy (8) or thymectomy and irradiation (20) did not reduce susceptibility to Multi-Strep diabetes. Interpretation of this latter study is complicated by radiation-induced damage to the testes which alone reduces susceptibility by reduction of testosterone levels (10, 11, 21).

The susceptibility of inbred strains to Multi-Strep diabetes has permitted attempts to ameliorate the diabetes by the transplantation of syngeneic islets. It was found that islet transplantation in C57BL/KsJ mice could restore euglycemia and, as in the high-dose SZ-induced diabetes, the recipients were permanently cured (22, 23). However, in contrast to mice rendered diabetic after high-dose SZ, insulitis and hyperglycemia recurred in the Multi-Strep transplant recipients when three additional subdiabetogenic doses of streptozotocin were given (23, 24).

There is, then, evidence for an immune involvement in the pathogenesis of diabetes after Multi-Strep. The results, however, are far from unequivocal, and in some mouse strains

(C57BL/KsJ) considerable evidence exists that the hyperglycemia induced by the Multi-Strep treatment may be largely or completely mediated by direct toxicity on the pancreatic beta cells (25, 26). There are strain differences in susceptibility to Multi-Strep which may, as in human diabetes, be associated with certain histocompatibility types. In the present study, we have evaluated the hypothesis that a cytotoxic immune response is involved in the pathogenesis of diabetes after Multi-Strep. Splenocytes, specifically cytotoxic for a rat insulin-producing cell line, were detected during the induction of Multi-Strep diabetes in a susceptible strain, and this cellular immune response was further characterized in vitro.

Methods

Suppliers. Male F1 hybrid C3D2 mice (male DBA/2J × female C3H/Tif) were purchased from Gl. Bomholtgaard (Ry, Denmark). Sprague-Dawley rats were obtained from a colony maintained at the Biomedical Center, Uppsala University, Uppsala, Sweden. NMRI mice were obtained from Anticimex (Sollentuna, Sweden). A clone of a rat insulinoma cell line (RIN) tht produced substantial amounts of insulin (clone 5F [27]) was obtained through the generosity of Dr. Ake Lernmark (Hagedorn Research Laboratory, Gentofte, Denmark). A human keratinocyte cell line (SCC 15 [28]) was generously provided by Dr. Magnus Nilsson (Institute of Cell Research, Uppsala University, Uppsala, Sweden). A fibroblast cell line (3T3) was obtained from Dr. Tibor Barka (Department of Anatomy, Mt. Sinai School of Medicine, New York). Culture medium (RPMI 1640), glutamine, penicillin-streptomycin and trypsin-EDTA stock solutions, calcium-magnesium-free Hank's balanced salt solution (CMF), and fetal bovine serum were obtained from Flow Laboratories Inc. (Irvine, Scotland). Culture dishes, sterile pipettes, and tubes were obtained from A/S Nunc (Kamstrup, Denmark). SZ (lots 1180K and 60,273-5) was generously provided by Dr. William Dulin (Upjohn Co., Kalamazoo, MI). $Na_2{}^{51}CrO_4$ (specific activity, 250–500 mCi/mg Cr) was purchased from Amersham Corp. (Amersham, England). Mitomycin-C, HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid), and 2-mercaptoethanol were obtained from Sigma Chemical Co. (St. Louis, MO).

Induction of diabetes. Young adult male C3D2F1 mice (25–30 g body wt) were randomly divided into groups. One group received one intraperitoneal injection of SZ per day for five days (40 mg/kg per d in 200 μl citrate buffer, pH 4.5 [2]). These mice will be referred to as the Multi-Strep group. Some mice received only a single high dose of SZ (200 mg/kg i.p.). Control mice received similar injections of citrate buffer only. The time after the induction of diabetes was determined from the day of the first injection of SZ. The serum glucose concentrations of the mice were determined only at the time of sacrifice in serum obtained from the cervical stump after decapitation. The serum glucose concentration was assayed with an automated glucose analyzer (Beckman Instruments Inc., Fullerton, CA).

Transplantation of RIN cells. As a positive control, some mice were injected intraperitoneally with a suspension of RIN cells prepared as described below. After resuspension in complete medium, 5×10^6 cells were washed three times in Hank's balanced salt solution and injected intraperitoneally in a volume of 200 μl through a 23-gauge (ga) needle. Some mice received a second injection of 2×10^6 cells 14 d after the first injection.

Maintenance of RIN cells. Clone 5F of this rat insulinoma cell line was maintained in tissue culture in RPMI medium 1640 supplemented

with 10% heat-inactivated fetal bovine serum, glutamine (1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and HEPES (10 mM). This medium will be hereafter designated complete medium. The RIN cells were passaged by detachment of the cells from the culture surface by the addition of trypsin-EDTA in CMF. The single cell suspension was rinsed in complete medium and either replated on new dishes or used as target or stimulator cells in the experiments to be described.

^{51}Cr labeling of target cells. The technique employed for the labeling of the cells to be used as targets in the cytotoxicity assay was similar to that described for the ^{51}Cr labeling of lymphocytes (29). The cells were dissociated with trypsin-EDTA in CMF, washed once in complete medium, resuspended in ~0.5 ml of complete medium, and incubated for 90 min in a CO_2 incubator (37°C in a humidified atmosphere of 5% CO_2 in air). They were resuspended in 8–9 ml of complete medium and counted in a conventional hemocytometer. A volume of the suspension corresponding to the number of cells required for a given experiment was transferred to a round-bottomed culture tube and the cells were pelleted by centrifugation (200 g for 5 min). The cells were resuspended in 300 μl of complete medium containing Na^{51}CrO$_4$ (20 cpm/cell) and returned to the CO_2 incubator. After a 90-min incubation, the labeled cells were washed three times in complete medium and resuspended at a final concentration of 2 × 10^5 cells/ml. Cells of the SCC 15 human keratinocyte line and the 3T3 cells were labeled in an identical manner. Sprague-Dawley rat and normal syngeneic C3D2F1 and allogeneic NMRI mouse lymphocytes were prepared as described below and labeled as described for the RIN cells except that the initial 90-min incubation was deleted. Some NMRI mouse and Sprague-Dawley rat lymphocytes were stimulated in vitro by culture in complete medium for 3 d in the presence of concanavalin A (ConA; 5 μg/ml; Pharmacia Fine Chemicals, Uppsala, Sweden) before ^{51}Cr labeling.

Splenocyte isolation. The spleens were removed from control and experimental mice and floated in 1–2 ml complete medium. Small holes were made in the capsule with a 23-ga needle and the splenocytes were flushed from the parenchyma by injection of complete medium slowly into the tissue through a 27-ga needle (30). The injection of ~20 ml was required to recover most of the cells, as evidenced by the blanching of the organ as the erythrocytes were expressed. The erythrocytes were lysed by suspension in ammonium chloride (0.17 M) for 10 min at 4°C and the macrophages were partially depleted by adherence onto tissue culture plates in the CO_2 incubator for 60 min in complete medium. By this technique, in our preliminary studies ~8 × 10^7 splenocytes were obtained from a single mouse spleen with a viability of 98%+ as determined by trypan blue dye-exclusion tests.

Cytotoxicity assay. Splenocytes from age-matched control (CT) and experimental (Multi-Strep or transplanted) (EXP) mice were always assayed in parallel. The splenocytes were diluted to the required concentration with complete medium. Since the target cells were used at 2 × 10^5/ml, splenocytes at 4 × 10^7/ml yielded an effector/target (E/T) ratio of 200. The splenocytes were serially diluted for E/T ratios of 100, 50, 25, 12.5, and 6.25. Splenocytes (100 μl) and ^{51}Cr-labeled target cells (100 μl) were mixed together in 11 × 50 mm plastic culture tubes which did not permit attachment of the target cells. The cells were pelleted together by centrifugation (400 g for 10 min) and returned to the CO_2 incubator for 5 h. Maximum release (MAX) was determined in tubes containing only target cells from which the supernatant medium was aspirated after centrifugation and replaced with 200 μl of Triton X-100 (0.3% vol/vol in distilled water). Basal release (BAS) was determined from tubes in which 100 μl of complete medium was added instead of a splenocyte suspension. After the 5-h

incubation, the tubes were recentrifuged, 100 μl of the supernatant fluid was removed, and the radioactivity was quantitated in a gamma counter (LKB-Wallac). Six replicates of each group were assayed. The mean±SEM counts per minute was calculated for each group. No lysis was said to be present if the mean counts per minute of the group being examined were not significantly greater than BAS at the 5% level or greater (one tailed t test, P ≤ 0.05). The lysis was calculated as follows and expressed as percent lysis:

$$[(cpm_{(CT \text{ or } EXP)} - cpm_{(BAS)})]/[(cpm_{(MAX)} - cpm_{(BAS)})] \times 100.$$

Stimulation of splenocyte proliferation in vitro. The method to be described was adapted from that used as the initial step in the establishment in culture of antigen-specific, cytotoxic T splenocyte clones (31, 32). Splenocytes (without lysis of the erythrocytes or removal of the macrophages by attachment) from control and Multi-Strep mice 10 d after the first dose of SZ were used as responding cells (final concentration, 2.5 × 10^6 cells/ml). To prevent mitosis, RIN cells were incubated at a concentration of 2–5 × 10^6 cells/ml in serum-free RPMI medium which contained mitomycin (25 μg/ml) and incubated in aluminum foil-wrapped tubes in the CO_2 incubator for 20 min. The mitomycin was inactivated by the addition of complete medium. The cells were washed three times in complete medium and resuspended to a final concentration of 10^5 cells/ml. The splenocytes and RIN cells were cultured together in a volume of 10 ml in T-flasks which contained complete medium supplemented with 2-mercaptoethanol (5 × 10^{-5} M) as a reductant and T cell growth factor (TCGF; interleukin-2, 5% vol/vol of a stock preparation). The cytotoxic response was evaluated by the ^{51}Cr-labeled RIN cell cytotoxicity assay. Aliquots of the control and experimental splenocytes were assayed before culture and after 5 d in vitro.

TCGF was prepared by the incubation of rat spleen cells at 5 × 10^6 cells/ml in Dulbecco's modified Eagle's medium containing 2-mercaptoethanol (5 × 10^{-5} M), HEPES (10 mM), bovine serum albumin (0.02%), and ConA (5 μg/ml) for 24 h. The cell-free culture supernatant was precipitated with ammonium sulfate at 80% saturation for 4 h at 4°C. The precipitate was collected and dialyzed overnight against 0.15 M NaCl-HEPES (10 mM) and passed over a 5 × 100 cm Sephadex G100 column equilibrated with the same buffer. The postalbumin fractions containing TCGF were pooled and assayed for growth promoting activity as previously described (33).

Histology. The pancreata of all of the mice killed to obtain splenic splenocytes were fixed in Bouin's solution and embedded in paraffin. Several sections were mounted on glass slides and stained with hematoxylin and eosin. After coding was done, the degree of insulitis was determined blindly by two independent observers according to the grading system previously published (34).

Statistical analyses. Differences between groups were established by the t test for unpaired samples.

Results

The ^{51}Cr-labeled RIN cell assay proved to be highly reproducible and sensitive (Table I). The RIN cells from 15 different passages were used and could be labeled relatively uniformly. The counts in the MAX tubes represented 47.8±0.6% of the total counts added in the assay, i.e., a 95.6±1.2% recovery based on radioactive counts. The basal lysis in the absence of splenocytes was quite high, suggesting that the RIN cells are leaky with regard to chromium. Despite the xenogeneic nature

Table I. ^{51}*Cr-Labeled RIN Cell Cytotoxicity Assay*

Parameter	Mean±SEM	Range
Maximum lysis (*cpm*)	11,835±880	7,936–13,905
Basal lysis (*% Max*)	18.4±0.6	13.1–22.6
Control lysis (%)	0.3±0.2	0–2.2
Intra-assay variation (%)	2.1±0.1	0.5–3.9

Data from 52 assays, mean±SEM.
Maximum lysis, 47.8±0.6% of the total counts added.

of the RIN cells, the nonspecific lysis by the splenocytes obtained from the control mice was remarkably low. In only 5 of 52 assays were the counts in the presence of control splenocytes significantly greater than basal. The mean lysis by the groups of control lymphocytes in the experiments described below was never significantly greater than zero. The extremely low intra-assay variability (2.1±0.1% of the counts per minute) permitted sensitive detection of low levels of specific lysis.

The changes in the cell-mediated cytolysis as measured by the ^{51}Cr-labeled RIN cell assay after Multi-Strep are compared with changes in serum glucose in Fig. 1. No specific cytolysis by the splenocytes from the Multi-Strep mice could be detected on the day after the last of the five doses of SZ (day 5). By day 8 ~5% of the ^{51}Cr-labeled RIN cells were specifically lysed by the Multi-Strep splenocytes ($P < 0.001$ compared with control splenocytes). At this time no change in serum glucose could be detected between the control and Multi-Strep mice. The level of the cytotoxic response peaked ~10 d after Multi-Strep, when 8.8±0.8% of the RIN cells were lysed. By
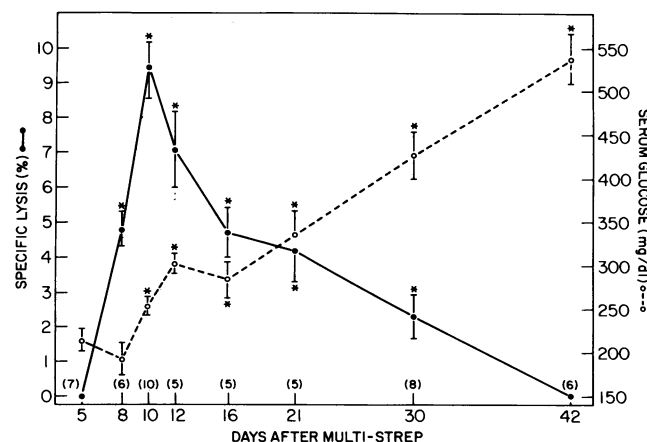

Figure 1. Serum glucose and specific lysis by splenic lymphocytes from Multi-Strep mice at various times after the first injection of SZ. The number of mice examined at each time point is given in parentheses. The serum glucose of CT mice (178±3 mg/dl, $n = 59$) did not change significantly during the 6-wk observation period. The bars indicate ±1 SEM. *Different from CT mice, $P < 0.01$ by t test.

this time, there was a small but statistically significant ($P < 0.01$) increase in serum glucose compared with that for age-matched vehicle-injected control mice. Subsequently, there was a continued rise in serum glucose concentration which reached a maximum of 539±29 mg/dl at the end of the observation period (42 d). However, the cytotoxic response fell significantly ($P < 0.005$) by day 16 and was undetectable in all six mice examined 42 d after the first dose of SZ.

Sections of the pancreata of these mice were examined for the presence of insulitis. The results are summarized in Table II. No insulitis could be detected on day 5. Two of the six mice examined on day 8 showed mild insulitis. By 10 d after Multi-Strep, however, most of the pancreata showed severe insulitis. This persisted through day 21, after which most of the pancreata examined exhibited the marked loss of islets characteristic of the pancreata of severely diabetic mice.

Since the peak of the cytotoxic response was detected at day 10, all subsequent experiments were performed with splenocytes obtained on that day. The cytotoxic response of the splenocytes from the Multi-Strep mice appeared to be specific for RIN cells. As detailed in Table III, neither normal syngeneic nor allogeneic mouse splenocytes nor xenogeneic rat splenocytes were specifically lysed by the Multi-Strep splenocytes. Furthermore, allogeneic or xenogeneic lymphoblasts (ConA stimulated) were not specifically affected by the Multi-Strep splenocytes. Other established cell lines, including a fibroblastoid (3T3) and an epithelial (human keratinocyte) cell line, were also not lysed specifically by the Multi-Strep splenocytes.

The cytotoxic response of the Multi-Strep splenocytes was not caused simply by the diabetic environment or by exposure to SZ. Splenocytes from mice in which diabetes had been induced by a single high dose of SZ lysed the ^{51}Cr-labeled RIN cells only slightly (Table IV). Splenocytes of five of the

Table II. Changes in Pancreatic Insulitis After Multi-Strep

Days after Multi-Strep	*n*	Insulitis			
		A	B	C	D
5	7	7			
8	6	4	2		
10	10		1	7	2
12	5			5	
16	5	1	1	3	
21	5		3	1	1
30	8	1			7
42	6	2			4

Numbers of pancreata showing each degree of insulitis are given. Insulitis scale (22): A, normal islet structure; B, some infiltration by mononuclear cells; C, heavy infiltration into a large number of islets; D, diabetic appearance (only a few small islets remaining).

Table III. Specificity of Cytotoxicity by Splenocytes 10 d After Multi-Strep

Target cell	Specific lysis
Lymphocytes	
Mouse	
(C3D2)	0
(NMRI)	0
(NMRI) ConA	0
Rat	
(S-D)	0
(S-D) ConA	0
3T3 cells	0
SCC-15 cells	0
RIN cells	9.2±1.0%

Data are from 12 assays. Each target was exposed to control and Multi-Strep splenocytes from five mice in separate assays. RIN cells provided a positive control in each assay. There was no significant lysis of any target by the control splenocytes.

eight mice tested exhibited no specific lysis of the RIN target cells. The mice receiving the single high dose of SZ had a significantly greater blood glucose level when sacrificed 10 d after the single injection than did the Multi-Strep mice.
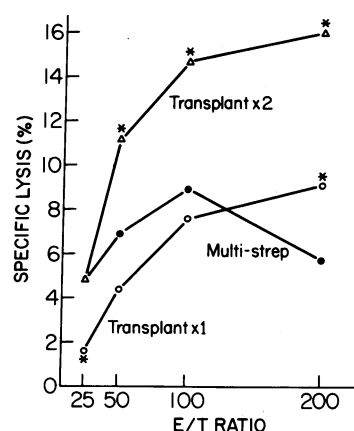
The absolute degree of cytolysis of the ^{51}Cr-labeled RIN cells appeared to be low. In order to establish an independent measure of the sensitivity of the assay, mice were injected intraperitoneally either once or twice with RIN cells and their splenocytes were isolated 10 d after the last injection. The dose response of the cytotoxicity of these splenocytes was compared with that after Multi-Strep (Fig. 2). There was an increase in the cytotoxicity of the Multi-Strep splenocytes as the E/T ratio was increased from 25 to 100, with a slight decrease in the cytolysis of the labeled RIN cells when the E/T ratio was increased to 200. Splenocytes from the mice that had received only one injection of RIN cells exhibited a cytotoxic response slightly lower than that of the Multi-Strep splenocytes. The degree of cytolysis was slightly greater by splenocytes from those mice that received two injections of RIN cells. However,

Table IV. RIN Cell Cytotoxicity by C3D2F1 Splenocytes 10 d after High- or Multiple Low-Dose SZ

Dose	Lysis	Serum glucose
		mg/dl
40 mg/kg × 5	9.0±1.1%	233±12
200 mg/kg × 1	0.8±0.5%*	381±43‡

Data are means±SEM (n = 8).
Different from 40 mg/kg × 5: *P < 0.001; ‡P < 0.01.

Figure 2. Specific lysis of RIN cells as a function of E/T ratio by splenic lymphocytes from Multi-Strep mice or mice previously transplanted once (× 1) or twice (× 2) with RIN cells. Transplanted mice received RIN cells (see Methods) 10 d before determination of specific lysis by the ^{51}Cr-labeled RIN cells cytotoxicity assay. Multi-Strep lymphocytes were examined 10 d after the first dose of SZ. The data are from five mice in each group. The SEM averaged ~18% of the mean in all groups. *Different from Multi-Strep lymphocytes, P < 0.01.

even when splenocytes from these mice were used, the specific cytotoxicity did not exceed 16±3%.

In order to support the hypothesis that the RIN cells were lysed by cytotoxic thymus-dependent splenocytes, splenocytes were isolated from control and Multi-Strep mice and cultured for 5 d with mitomycin-inactivated RIN cells in a medium supplemented with TCGF. The dose response of cytotoxicity by both control and Multi-Strep splenocytes was determined before and after culture (Fig. 3). Before culture, the splenocytes from the control mice produced no lysis of the ^{51}Cr-labeled RIN cells above basal release. As previously shown, the splenocytes from the Multi-Strep mice exhibited specific cytolysis of the RIN cells detectable at an E/T ratio of 12.5, reaching a
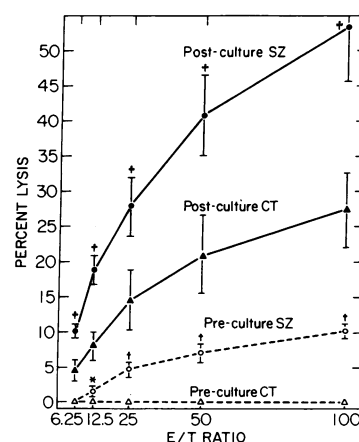

Figure 3. The effects of culture for 5 d in the presence of RIN cells and TCGF on the cytolysis by CT and Multi-Strep (SZ) splenocytes. Each point represents the mean from five experiments in each group before and after culture. The bars represent ±1 SEM. *Greater than CT, P < 0.01. †Greater than CT, P < 0.001.

maximum of $10.1\pm0.9\%$ at an E/T ratio of 100. As expected, after 5 d in culture with the xenogeneic RIN cell targets and TCGF, the control splenocytes produced a highly significant lysis of the ^{51}Cr-labeled RIN cells in a dose-dependent manner. However, the response of the Multi-Strep splenocytes was significantly greater than that of the control cells ($P < 0.001$), reaching $53.3\pm7.9\%$ at an E/T ratio of 100.

Discussion

These studies do not offer direct proof that cytotoxic lymphocytes are responsible for the destruction of the beta cells, but they do provide data that support the hypothesis that a cell-mediated anti–beta cell immune response is stimulated during the induction of diabetes after Multi-Strep. The time course of the development and regression of this response detected in vitro coincided with the course of the insulitis that is prominent in this model. An increased cytotoxicity could be detected before the onset of significant hyperglycemia. Several aspects of these studies require further elaboration.

It was unexpected that a cell-mediated response could be detected in mice against a xenogeneic target cell. RIN cells were initially used as targets because of the report that they bind human anti–islet cell surface antibodies (35), an observation that has been confirmed (36, 37). Recently, another example of cytotoxic T cells whose cytotoxic response was not major histocompatibility complex-restricted has been described (38). One can postulate that the class I major histocompatibility complex antigens on the surface of the RIN cells cross-react with the appropriate receptors on the mouse splenocytes. Since the RIN cells produce relatively large amounts of insulin and express the putative beta cell specific antigens for direction of both humoral and cell-mediated immune responses, they will provide an excellent tool to define further these processes. In this regard the RIN cells are superior to targets previously used in attempts to detect cell-mediated immune responses in diabetes. To our knowledge, there have been no previous attempts to demonstrate in vitro a cell-mediated anti–beta cell immune response in other animal models of diabetes. By the use of lymphocytes of diabetic patients as effectors, the cytotoxic response was examined by employing cells of a human insulinoma cell line as targets (39). These target cells, however, had lost the ability to secrete insulin, and no attempt was made to detect specific cell surface markers. In several other studies either isolated rat islets (40) or dispersed rat islet cells (41, 42) were the target cells. Few patients were examined in all of these studies, and, as in the present study, the levels of specific lysis were low. The inbred mouse proved to be an excellent model for these studies, since, unlike in the human, control splenocytes can be obtained from age-matched, genetically identical animals. As noted, however, the cytolysis by the control splenocytes was always low and the failure to have used these controls would not have affected the interpretation of the data to any significant degree.

The maximum cytolysis of the RIN cells was <10%. This is in contrast with cytolysis as high as 80–90% in many well-characterized cell-mediated cytotoxicity assay systems. The numbers of specific effector lymphocytes in the spleen cell preparations we used may have been very low. The detectable cytotoxic response was equally low after the transplantation of RIN cells into normal C3D2F1 mice, suggesting that the immune response detected during the Multi-Strep, although low, may be pathologically meaningful. Actually, the detection of any cell-mediated response against the RIN cells may be significant, since several groups have previously demonstrated a deficient cell-mediated immune response in uncontrolled diabetic humans (43) and in experimentally diabetic animals (44, 45). The decrease in the level of the cell-mediated anti-RIN cell response in the Multi-Strep mice may have, in part, been due to the onset of severe insulinopenia in these animals after 21 d (3). The destruction of the source of the antigenic stimulus, the beta cells, would also have been expected to result in a gradual decrease in the immune response. Another possible explanation for the low cytotoxicity may be that the RIN cells, although leaky with regard to ^{51}Cr-labeling (18% basal release), may be difficult to lyse. This explanation is unlikely, however, in light of the previous studies of antibody-mediated lysis of these cells (35–37) in which >50% lysis was frequently detected.

The time course of the detectability of a cytotoxic response after Multi-Strep provides a possible explanation for the failure of most previous attempts to transfer passively diabetes from Multi-Strep to syngeneic control or nude mice (14, 16, 17). First, the cytotoxic response was low, suggesting that there is only a very low number of specific cytotoxic T cells in the splenocyte preparations. Second, in most of these reports, the splenocytes were obtained after the onset of severe hyperglycemia. Thus, if our studies are applicable to other strains of mice, the cytotoxic immune response probably had abated before the passive transfer was attempted. This hypothesis is supported by the experiments reported recently with Bio-Breeding/Worcester rats. Stimulation in vitro by ConA was required before splenocytes from newly diabetic rats could transfer the diabetes after injection into susceptible hosts (46).

The stimulation of the cytotoxicity after culture with RIN cells and TCGF not only supports the hypothesis that thymus-dependent splenocytes are responsible for the lysis of the target, but, more important, suggests that the proliferation of these cytotoxic cells can be stimulated and maintained in vitro. TCGF seems to be a relatively specific stimulator of the cytotoxic T splenocytes (31, 32), although a recent study has suggested that natural killer cells may also be stimulated by the presence of very high concentrations of this growth factor (47). Presumably, the increase in cytotoxicity after culture reflects an increase in the number of specific effector cells. If the proliferation of the cytotoxic splenocytes could be maintained beyond the 5-d period examined in this study, it might become possible to establish a cloned anti–RIN cytotoxic cell line. Such a cell line would be ideal in establishing, by passive

transfer, the actual role of these splenocytes in the pathogenesis of diabetes in this animal model. A clone or clones of T cells would also be invaluable in identifying and characterizing the specific beta cell antigenic determinants which may be, in part at least, species independent. Finally, specific antibodies could be developed against cell surface markers unique to this clone or clones. By the use of such antibodies, the cytotoxic splenocytes could be selectively destroyed and, if the cytotoxic response is important in the pathogenesis of the beta cell destruction, diabetes would be prevented or its progression blocked in the earliest stages.

Acknowledgments

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