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L3T4 and Lyt-2 T cells are both involved in the generation of low-dose streptozotocin-induced diabetes in mice

GUDRUN KANTWERK, S. COBBOLD*, H. WALDMANN* & H. KOLB *Diabetes-Research Institute, University of Düsseldorf, Düsseldorf, FRG and *Department of Pathology, University of Cambridge, Cambridge, UK*

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SUMMARY

In order to determine the role of different T lymphocyte subsets in the pathogenesis of low-dose streptozotocin (LD-Sz) induced diabetes, we treated mice with Sz together with repeated injections of rat monoclonal antibodies (MoAb) with specificity towards the mouse T cell differentiation markers L3T4 ('helper/inducer' T cells and some macrophages), Lyt-2 ('cytotoxic/suppressor' T cells and NK cells) and Thy-1 (pan T lymphocytes). Treatment depleted target cells in peripheral blood and spleen; decreased the ability of spleen cells to respond to mitogens; and, in the case of depletion of the L3T4 T cell subset, prevented a humoral immune response to SRBC. Treatment with MoAb against either of the two T cell subtypes could protect from hyperglycaemia and loss of body weight, suggesting that both T cell subsets were implicated in the development of LD-Sz induced diabetes. Immunocytochemical analysis of pancreatic sections showed that both L3T4$^+$ and Lyt-2$^+$ cells participated in islet infiltration together with macrophages. Treatment with MoAb markedly reduced islet infiltration by both L3T4$^+$ and Lyt-2$^+$ cells but not by macrophages. The suppressive effect of MoAb against either L3T4 or Lyt-2 on diabetes development suggests that the pathomechanism involved is different from that in experimental autoimmune neuritis and adjuvant arthritis where Lyt-2 cells are not involved.

Keywords type I diabetes streptozotocin autoimmunity monoclonal antibodies immunotherapy

INTRODUCTION

The human 'autoimmune' disease type I diabetes is characterized by infiltration of the pancreatic islets with lymphocytes and macrophages (insulitis) (Gepts & LeCompte, 1981). Treatment with the immunosuppressant cyclosporin A is able to stop progression of the disease (Feutren *et al.*, 1986; Stiller *et al.*, 1984). The low-dose streptozotocin (LD-Sz) induced diabetes in mice (Like & Rossini, 1976) serves as an animal model of type I diabetes. The diabetogenic properties of Sz were discovered by Rakieten, Rakieten & Nadkarni (1963): one single large dose causes specific, acute degeneration of β-cells in Langerhans' islets of the pancreas and chronic hyperglycaemia whereas five daily injections of smaller doses of Sz produce a delayed but progressive increase in blood glucose and islet infiltration with lymphocytes and macrophages, accompanied by beta-cell necrosis and hypoinsulinaemia (Like & Rossini, 1976). In 1978 Rossini *et al.* demonstrated the active

Correspondence: Professor H. Kolb, Diabetes-Research-Institute, Auf'm Hennekamp 65, 4000 Düsseldorf 1, FRG.

participation of lymphocytes in the development of LD-Sz diabetes: treatment with anti-lymphocyte serum protected LD-Sz treated mice from hyperglycaemia. With the development of rat monoclonal antibodies (MoAb) against mouse T lymphocyte differentiation markers (Galfre, Milstein & Wright, 1979; Cobbold *et al.*, 1984) it now becomes possible to investigate the role of the two major lymphocyte subsets in the pathogenesis of LD-Sz diabetes.

In this report, we show that treatment with rat MoAb specific towards the antigens Thy-1 (marker for all T cells), L3T4 (marker for T cells of the 'helper'-phenotype and some macrophages) or Lyt-2 (marker for T cells of the 'cytotoxic/suppressor'-phenotype and NK cells) could suppress the development of LD-Sz diabetes in mice.

MATERIAL AND METHODS

Mice. Male C57BL/6, 9-week-old mice (Bomholtgard, Ry, Denmark) received a standard diet ('ssniff-M', Ssniff, Soest, FRG) and drinking water with 50 mg/l oxytetracycline (Serva, Heidelberg, FRG) *ad libitum.*

Streptozotocin (Sz). Sz (Boehringer, Mannheim, FRG), dissolved in 25 mM sodium-citrate pH 4 just before use, was given for 5 consecutive days (d0–d4) intraperitoneally, 40 mg/kg body weight.

Monoclonal antibodies. Rat monoclonal antibodies against the mouse T cell markers Thy-1 (YTS 154·7), L3T4 (YTS 191·1·2) and Lyt-2 (YTS 169·4·2) were prepared as described previously (Galfre *et al.*, 1979; Cobbold *et al.*, 1984). Each antibody solution 0·2 ml per animal; (5 mg/ml) was injected intravenously on d0 and d4 (6 h before Sz administration) and intraperitoneally on d10. For immunohistochemical analysis in addition the following antibodies were used: natural-killer-cell specific rabbit anti-asialo GM1 (Wako Chemicals GmbH, Neuss, FRG); macrophage specific MoAb F4/80 (kind gift of S. Gordon, Oxford, UK).

Experimental groups. One group of mice ($n = 15$) was treated with Sz alone, four other groups ($n = 10$) received in addition to Sz the different T-lymphocyte specific MoAb: (1) anti Thy-1, (2) anti Lyt-2, (3) anti L3T4, (4) anti Lyt-2 and anti L3T4 together. Four further groups ($n = 5$) were treated with the MoAb alone, and one group ($n = 5$) remained untreated until d28, at which time all five groups were immunized with SRBC (sheep red blood cells).

Experimental parameters. Blood glucose was measured by the hexokinase method using an automatic analyser (Boehringer Glucoquant, LKB Ultrolab System, Bromma, Sweden). Histological investigation of pancreata and spleen was performed on days 10 or 28 of the experiment.

Histological investigation. Frozen tissue sections (5 μm, Frigocut 2700, Reichert-Jung, Nussloch, FRG) and blood smears ($n = 5$, from blood collected at day 28 from the retroorbital venous plexus) were stained by an immunohistochemical method briefly summarized as follows: (1) fixation in acetone −20°C, 5 min; (2) airdrying; (3) fixation in ethanol −20°C, 1 min; (4) washing in PBS (phosphate buffered saline) 10 mM, pH 7·4, (5) incubation with PBS containing 5% RNS (rabbit normal serum); (6) washing in PBS; (7) incubation with MoAb to the different T cell subsets and natural-killer-cells, 20 μg per ml PBS containing 1% bovine serum albumin (BSA) and 0·1% NaN_3, F4/80 cell culture supernatant 1:20, +4°C, 18 h; (8) washing in PBS; (9) incubation with rabbit anti rat IgG-biotin labelled (Vectastain ABC kit, Vector-Laboratories, Camon, Wiesbaden, FRG), 30 μg per ml PBS containing 5% heat inactivated mouse normal serum (MNS); (10) washing in PBS; (11) incubation with avidin-biotin-HRP (horse radish peroxidase) complex (Vector-Laboratories); (12) washing in TRIS-HCl buffer 50 mM, pH 7·6; (13) incubation with substrate solution; 0·5 mg 3'3 diaminobenzidine tetrahydrochloride (EGA-Chemie, Steinheim/Albuch, FRG) per ml TRIS-HCl buffer containing 0·025% H_2O_2, RT, 10 min.; (14) counterstaining in Mayer's haematoxylin; (15) dehydration and mounting in 'Eukitt'-medium (Klees, Düsseldorf, FRG).

Mitogenic stimulation of spleen cells. Cells were isolated at day 28 and incubated with mitogens as described previously (Kolb *et al.*, 1985). Briefly, spleen cells ($0·2 \times 10^6$ in 200 μl modified RPMI 1640) were incubated with concanavalin A (Con A), pokeweed-mitogen (PWM) and phytohaemagglutinin (PHA) at 0·5, 1·25 and 2·5 μg/ml for 72 h. ^{3}H-Thymidine was added for the last 18 h, and the radioactivity incorporated was measured after harvesting the cells.

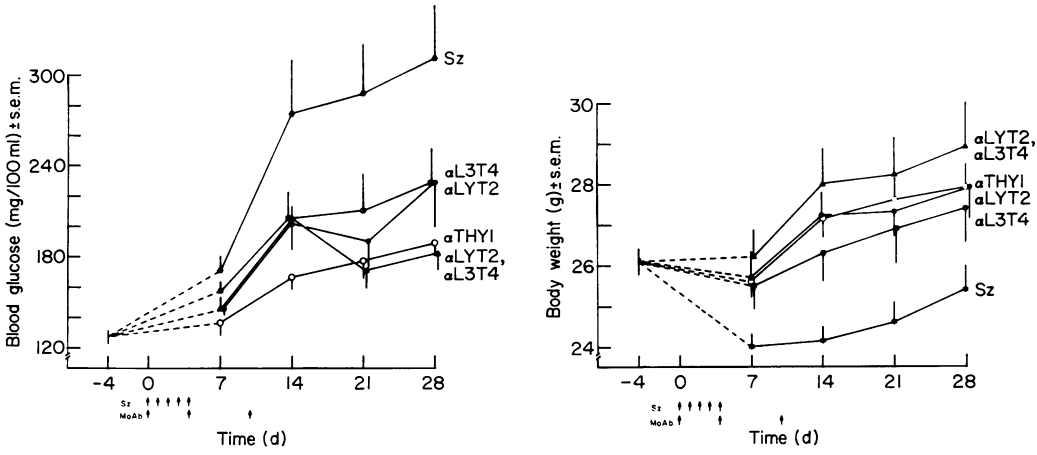

Fig. 1. Effect of treatment with MoAb to Thy-1, L3T4, Lyt-2 on diabetes development after LD-Sz treatment. (a) Effect on hyperglycaemia; animals treated with MoAb alone showed mean values of about 155 mg/dl on day 28 which is in the normal range. (b) Effect on body growth; during a period of 4 weeks untreated mice as well as animals treated with MoAb alone showed an increase of body weight of about 2–3 g.

Measurement of the humoral immune response. Mice treated with the different MoAb or Sz and untreated mice ($n = 5$) were immunized intraperitoneally on day 28 with 0·2 ml 10% sheep red blood cell (SRBC) suspension solution (Behring-Werke, Frankfurt, FRG). On day 38, blood from each animal was collected, the serum was separated and heat-inactivated (56°C, 30 min), 50 μl of serum was incubated with 200 μl β-mercaptoethanol (β-ME) 0·1 M in PBS with 2% mouse normal serum. The direct haemagglutination assay with SRBC was performed in round bottomed microtitreplates (Falcon): serial dilutions (100 μl) of β-ME and untreated sera of each animal were incubated at RT with 100 μl or 1% SRBC. Haemagglutination titres were determined after 60 min.

RESULTS

Effect of antibody treatment on diabetes development. Blood glucose levels in mice treated with Sz alone increased during the 28 days of the experiment from about 130 mg/dl up to about 310 mg/dl (Fig. 1a). Animals treated with the specific antibodies against T lymphocytes or their subtypes in addition to Sz showed significantly reduced hyperglycaemia: animals which had been treated with MoAb to L3T4 or with anti Lyt-2 MoAb showed blood glucose values of about 220 mg/dl on day 28 ($P < 0·05$, Student's t-test, one-tailed). Mice treated with MoAb against Thy-1 or the combination of MoAb to L3T4 and Lyt-2 showed complete inhibition of hyperglycaemia ($P < 0·025$ and 0·05), (Fig. 1a). After treatment with Sz, mice lost weight. This was not observed in mice given the different MoAb in addition to Sz. These mice had a normal weight gain of about 2–3 g over a period of 4 weeks (Fig. 1b).

Islet histology. At days 10 and 28 of the experiment, an immunocytochemical analysis of cells infiltrating pancreatic islets was performed. As shown in Table 1 LD-Sz causes infiltration of islets with mononuclear cells positive for Thy-1, L3T4, Lyt-2, F4/80 but not with asialo-Gm1 positive cells. An additional observation is the occurrence of asialo-GM1 positive cells throughout the exocrine tissue at day 10.

Treatment of LD-Sz receiving animals with monoclonal antibodies resulted in a reduction of islet infiltration which is most pronounced when analysing for more severely infiltrated islets (Table 1). Administration of antibody to L3T4 reduced islet infiltration by L3T4$^+$ as well as by Lyt-2$^+$ cells. The same result was found after treatment with Lyt-2 antibody. Interestingly, the heavy

Table 1. Immunocytochemical analysis of insulitis after low dose streptozotocin and monoclonal antibody treatment

Treatment	Day of analysis	Antibody specificity	Islets analysed* (*n*)	Islets (*n*) with stained infiltrating cells		% Islets with ≥4 stained infiltrating cells
				1–3	≥4	
None		Thy-1	15	2	0	0
		L3T4	16	2	0	0
		Lyt-2	11	0	0	0
		F4/80	8	7	0	0
		asialo GM1	7	0	0	0
LD-Sz	10	Thy-1	15	5	7	47
		L3T4	14	3	2	14
		Lyt-2	17	3	2	12
		F4/80	4	3	1	25
		asialo GM1†	7	2	0	0
	28	Thy-1	22	9	7	32
		L3T4	19	7	2	11
		Lyt-2	12	2	3	25
		F4/80	12	3	8	67
		asialo GM1	12	1	0	0
LD-Sz anti-Lyt-2 anti-L3T4	10	Thy-1	20	8	0	0
		L3T4	14	1	0	0
		Lyt-2	27	7	0	0
		F4/80	7	3	3	43
		asialo GM1	7	1	0	0
	28	Thy-1	14	4	1	7
		L3T4	11	2	0	0
		Lyt-2	9	0	0	0
		F4/80	8	3	4	50
		asialo GM1	8	2	0	0
LD-Sz anti-Thy-1	10	Thy-1	15	4	3	20
		L3T4	29	2	0	0
		Lyt-2	23	6	2	9
	28	Thy-1	12	2	0	0
		L3T4	12	2	0	0
		Lyt-2	17	2	0	0
LD-Sz anti-L3T4	10	Thy-1	16	9	2	13
		L3T4	16	4	0	0
		Lyt-2	14	3	1	7
	28	Thy-1	17	7	2	12
		L3T4	18	3	0	0
		Lyt-2	19	4	1	5
LD-Sz anti-Lyt-2	10	Thy-1	20	4	3	15
		L3T4	23	1	0	0
		Lyt-2	22	3	1	5
	28	Thy-1	15	5	5	33
		L3T4	13	2	1	8
		Lyt-2	14	3	0	0

* Number of islets found on tissue sections of five animals respectively.
† The amount of asialo GM1$^+$-cells within the exocrine was increased on day 10 in LD-Sz treated mice:

Treatment		Total counts of asialo GM1$^+$-cells within the exocrine tissue (~5 × 5 mm resp.)
LD-Sz	day 10	71
	day 28	20
LD-Sz anti-L3T4 anti-Lyt-2	day 10	14
	day 28	13
untreated		1

infiltration of islets by F4/80 positive macrophages was not inhibited after treatment with antibodies to L3T4 and Lyt-2.

Immunosuppressive effect of antibody treatment. On day 10 of the experiment histological investigation of cryostat sections of spleens demonstrated a near complete depletion of T cell subsets from T-lymphocyte areas in the white pulp of animals treated with the different MoAb (Table 2). Analysis of peripheral blood also showed specific depletion of subsets by MoAb (Fig. 2).

Table 2. Distribution of T cell subsets in the T cell area of the white pulp in the spleen on day 10 of the experiment*

Treatment	Approximate amount of cells (%)†		
	Thy 1$^+$	L3T4$^+$	Lyt 2$^+$
None	95	40–50	40–50
anti Thy 1	<2	<2	<2
anti L3T4	40–50	<2	40–50
anti Lyt 2	40–50	40–50	<2

* On day 28 repopulation of spleens with both lymphocyte subsets could be observed in some animals approaching about 10–30% of the normal counts.

† Each value is derived from the analysis of spleen sections of five animals.

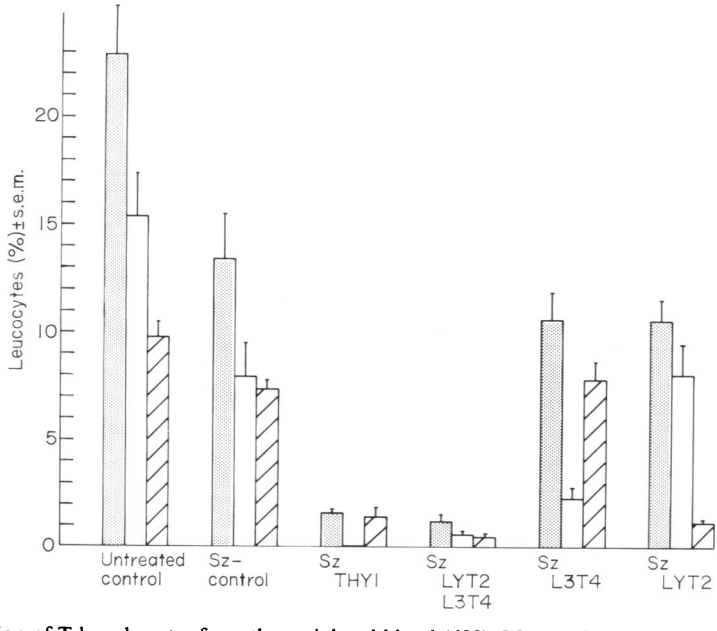

Fig. 2. Depletion of T lymphocytes from the peripheral blood (d28). Mean values were determined by three counts of 100 nuclear cells per stained blood smear. (▨) Thy-1$^+$-lymph; (□) L3T4$^+$-lymph; (■) Lyt-2$^+$-lymph.

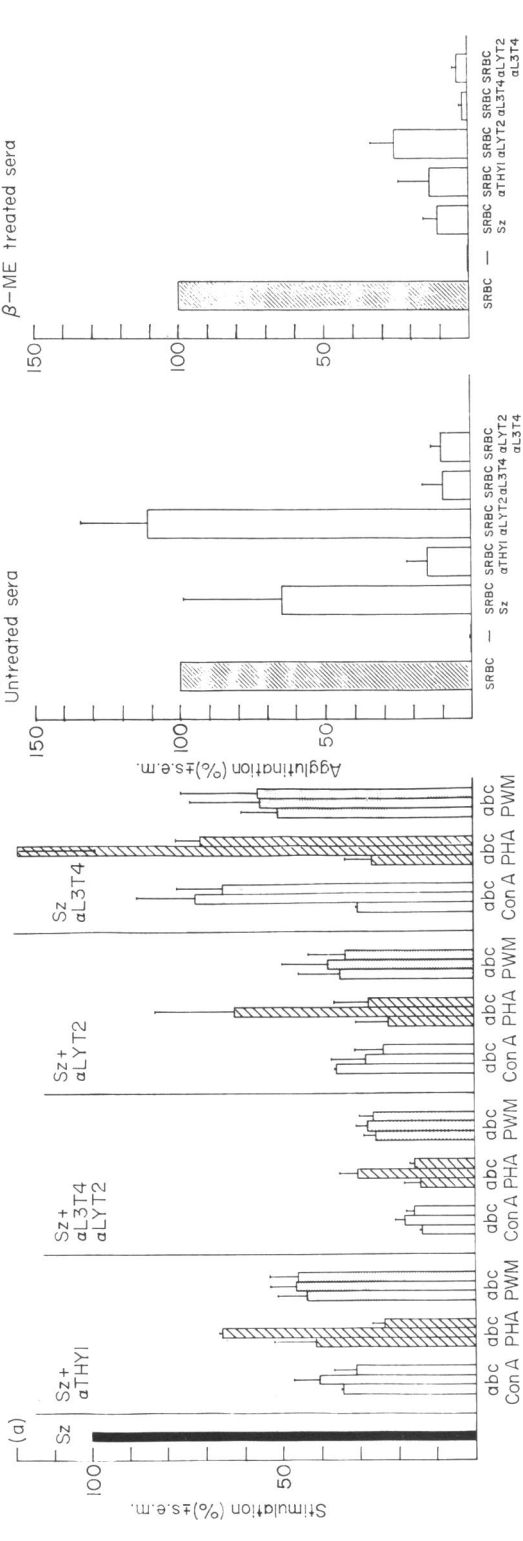

Fig. 3. Functional studies. (a) Stimulation of spleen cells with the mitogens Con A, PHA and PWM on day 28: the mean of triple values of ^{3}H-thymidine incorporation was determined. After stimulation, the indices of the Sz control group were considered as maximal stimulation = 100%: Con A, 0·5 μg/ml = 55·1; 1·25 μg/ml = 73·6, 2·5 μg/ml = 62·8. PHA, 0·5 μg/ml = 13·3, 1·25 μg/ml = 21·5, 2·5 μg/ml = 50·7. PWM, 0·5 μg/ml = 26·1, 1·25 μg/ml = 26·6, 2·5 μg/ml = 25·6. a, 0·5 μg/ml; b, 1·25 μg/ml; c, 2·5 μg/ml. (b) Humoral immune response to SRBC was estimated by determination of direct haemagglutination of sera of immunized animals with SRBC *in vitro*. The mean titre of SRBC immunized mice, given neither Sz nor MoAb was considered as maximal agglutination (100%) ($x \pm$ s.e.m.): untreated sera: 2765 ± 819; β-ME treated sera: 1819 ± 932.

Functional studies performed at day 28 revealed a decreased ability of spleen cells to respond to different mitogens (Fig. 3a); the depletion of the L3T4$^+$ -T cells almost completely prevented the humoral immune response to SRBC (Fig 3b).

DISCUSSION

The main finding of this study is that administration of each of the three MoAb caused an inhibition of diabetes development. The antibody treatment also prevented the retardation in growth seen after Sz administration and resulted in a reduction of pancreatic infiltration by T lymphocytes. Maximum inhibition of diabetes development was reached with either anti Thy-1 alone or anti L3T4 and anti Lyt-2 MoAb together. However, selective depletion of L3T4$^+$ or of Lyt-2$^+$ cells was sufficient to significantly suppress hyperglycaemia. This is reminiscent of observations in skin graft rejection where, depending on the circumstances, depletion of either L3T4 or Lyt-2 cells alone caused partial inhibition of graft rejection, whereas the combination of anti L3T4 plus anti Lyt-2 MoAb gave better graft preservation. The classical view derived from *in vitro* studies is that cytotoxic T-cells require the 'help' of CD4 positive 'helper' T cells for activation. However, recent evidence suggests that cytotoxic (Lyt-2) cells can act alone, i.e. independently of the presence of L3T4 cells (Cobbold & Waldmann, 1986; Guimezanes & Schmitt-Verhulst, 1985; Sprent & Schäfer, 1986).

Furthermore it was found that L3T4 cells may exert a cytolytic effect on target cells themselves (Sun & Wekerle, 1986). It may also be possible that L3T4$^+$ macrophages are responsible to some extent for beta-cell cytotoxicity. Experiments to prove the cytotoxic potential of L3T4$^+$ cells towards beta cells *in vitro* are in progress.

In this context it is of interest that the administration of silica particles caused suppression of diabetes development. The quartz dust is selectively taken up by macrophages and leads to their death or functional alteration (Oschilewski *et al.*, 1986). Electron microscopic studies revealed an early phase of 'single cell insulitis' characterized by the infiltration of macrophages in the islet. This was followed by 'multicellular insulitis' where the infiltrate consisted predominantly of lymphocytes (Kolb *et al.*, 1984).

Since the interference with L3T4 cells, or Lyt-2 cells or with macrophages (part of which may probably be L3T4 positive (Jefferies, Green & Williams, 1985)) alone is sufficient to largely prevent diabetes development in this model we assume that diabetes development is the result of a cascade of several immune reactions. This view is supported by immunocytochemical analysis of islet infiltration after LD-Sz described here for the first time. Using monoclonal antibody F4/80 we observed large numbers of macrophages within inflamed islets in addition to T lymphocytes of CD4 and CD8 subtypes. Most interestingly, NK-lymphocytes as defined by reactivity with asialo-GM1 antibody were not found among infiltrating cells but were observed dispersely infiltrating the exocrine tissue early during the disease. The exocrine pancreas may be involved in the induction phase of LD-Sz diabetes. As regards our assumption of a cascade of immune events it is important that depletion of animals of L3T4$^+$ and Lyt-2$^+$ cells did not inhibit infiltration of islets by macrophages (but did partially suppress infiltration of endocrine tissue by both L3T4 and Lyt-2 cells and also infiltration of exocrine tissue by NK-lymphocytes). This indicates that macrophage infiltration of islets occurs independently of lymphocytic infiltration and thus may be regarded as a separate event.

Our observation of two different lymphocyte subsets being involved in immune-mediated diabetes differs strikingly from other types of 'auto-immune' diseases. The depletion of cytotoxic lymphocytes prevented the induction of neither experimental allergic neuritis nor adjuvant arthritis in rats. In both models, depletion of total T cells was effective, implying that it is only the helper T cell which is responsible for disease development (Holmdahl *et al.*, 1985; Larsson *et al.*, 1985). The view that L3T4 and Lyt-2 subsets can contribute independently to diabetes development highlights the fact that the cellular components of the immune system are opportunistic: where Lyt-2 cells can see and respond to antigen they will do so. This appears to hold for graft rejection (Cobbold &

Waldmann, 1986), for GVHD, for marrow rejection (Cobbold, Martin & Waldmann, 1986; Cobbold *et al.*, 1986), and—as we show here—for immune-mediated diabetes.

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