

Lymphocyte transformation by insulin and insulin–zinc suspensions

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SUMMARY

The lymphocyte transformation response to the mitogen phytohaemagglutinin (PHA) was determined in 15 well controlled insulin-dependent diabetics (IDD) with a history of insulin allergy or an acute insulin allergy. There was no significant difference in the PHA response of IDD and normal subjects matched in respect of age and sex. The response of peripheral blood lymphocytes to insulin (Actrapid) and an insulin zinc suspension (Monotard) was also determined. Fifty-three percent of IDD gave a positive reaction to Actrapid. Monotard produced positive reactions both in IDD and normal subjects. In normal subjects, a close correlation between the stimulation indices of Monotard and PHA was found ($r=0.966$) suggesting that these stimulations depend on a common parameter namely, the reactivity to mitogens.

INTRODUCTION

A well known immunological side effect of insulin administration to diabetic subjects is insulin allergy. By far the most common of the many forms it may take (Goldstein, 1971), is a local skin reaction of the immediate or as described by Frei, Cruchaud & Vannotti (1965) of the delayed type. Whereas the humoral response to insulin (reviewed by de Shazo, 1978) has been investigated extensively, less attention has been paid to cell-mediated immune reactions to insulin in insulin treated diabetics.

It has been shown by *in vitro* experiments (Federlin, 1971; Halpern, Ky & Amache, 1967) that both bovine and porcine insulin can induce blastogenesis of peripheral blood lymphocytes from patients with immediate or delayed type hypersensitivity to insulin. More recent studies of Faulk, Girard & Welscher (1975) and MacCuish *et al.* (1975) using radioisotope techniques have confirmed these results and suggest a part of the B chain to be the epitope determining the cell-mediated reaction to insulin. Cell-mediated reactions to insulin *in vitro* have also been reported in untreated diabetics; they are rather common in insulin treated diabetics, although often without any evidence of allergy *in vivo* (MacCuish *et al.*, 1975).

At the beginning of the present study we were surprised by the distinct stimulation of peripheral blood lymphocytes from normal subjects by commercial insulin zinc suspensions (Lente-type insulins) and supposed therefore, that some additional factor was involved in lymphocyte transformation by insulin.

As many other agents, zinc is known to influence lymphocyte transformation possibly acting as a prosthetic group in many metalloenzymes (e.g. DNA polymerase). It induces lymphocyte transformation in serum containing media (Kirchner & Rühl, 1970; Loeb, 1974). Zinc also appears

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likely to be essential for PHA-induced lymphocyte transformation *in vitro* (Phillips & Azary, 1973). Addition of chelating agents leads to a decrease of ^3H -thymidine incorporation, which can be reversed by supplementation with zinc (Williams & Loeb, 1973). Insulin solutions prepared by ethanol extraction and further purification by recrystallization contain at least small amounts of zinc (British Pharmacopoeia, 1973). Many long-acting insulins are prepared by addition of zinc to obtain a prolonged effect (Schlichtkrull *et al.*, 1975). The zinc content of these preparations may therefore considerably influence lymphocyte transformation tests.

In the present study, we have investigated the *in vitro* response of peripheral blood lymphocytes to phytohaemagglutinin (PHA) and two different commercial insulin preparations (Actrapid and Monotard of Novo Industri A/S, Bagsvaerd) by measuring the ^3H -thymidine uptake. A group of 15 insulin-dependent diabetics (IDD) with acute insulin allergy (two) or a history of insulin allergy (13) and diabetics without insulin therapy as well as normal individuals, taken as control groups, have been studied.

MATERIALS AND METHODS

Subjects. In 15 well controlled insulin-dependent diabetic patients (IDD) with acute insulin allergy (two) or a history of insulin allergy (13) lymphocyte transformation studies have been performed. Their average age was 49.7 (with a range from 14 to 68). The non-specific response to phytohaemagglutinin (PHA) was compared with a group of 15 non-diabetic subjects sent to our outpatient clinic for different purposes (mainly penicillin allergy). The two groups were matched in respect of age and sex. The response to insulin preparations was compared with a control group of nine young and healthy volunteers as well as with a group of nine diabetic patients never treated with insulin before the tests (in all patients however insulin therapy was to be initiated subsequently).

Lymphocyte cultures. After sedimentation of heparinized venous blood the lymphocyte rich supernatant was withdrawn and the lymphocytes were separated from the remainder by density centrifugation on a Ficoll-Isopaque gradient (density 1.077). The cells were then washed twice with TC-Medium 199 (Difco Laboratories, Detroit, Michigan, USA) and resuspended in TC-Medium 199, buffered with 0.01 M HEPES buffer (Calbiochem, San Diego, California, USA) and supplemented with 20% autologous plasma respectively 20% AB serum, at a concentration of 1×10^6 cells per millilitre. The cell suspensions obtained by this method contained about 90% lymphocytes and more than 95% of the cells appeared to be vital by trypan blue exclusion.

One millilitre cultures were set up in triplicate with addition of 4 i.u./ml Actrapid or 4 i.u./ml Monotard, established as optimal by previous dose-response analysis. At the same time a portion of the lymphocytes was cultured together with 20 μg PHA in order to estimate the mitogenic response. The cultures were labelled with ^3H -thymidine for 18 hr at day 5 (for insulin stimulation) respectively at day 2 (for PHA stimulation). Cultures were then harvested and radioactivity of the filter paper discs was counted. Results were expressed as counts per minute (c.p.m.) and as a stimulation index: $\text{SI} = \text{c.p.m. with insulin} / \text{c.p.m. without insulin}$.

Insulin preparations. Actrapid and Monotard have been provided by Novo Industri A/S, Bagsvaerd (for composition see Table 1).

Statistical analysis. In comparative studies statistical significance of differences was assessed by a modified rank sum test (Riedwyl, 1973). Linear regression analysis was employed in correlation studies using the method of least-squares.

RESULTS

Mitogenic effect of PHA

The PHA response of peripheral blood lymphocytes in a group of 15 IDD with insulin allergy or a history of insulin allergy was compared with a group of 15 normal subjects matched in respect to age and sex. The results as presented in Table 2 showed a great variance. In medium supplemented with

Table 1. Composition of insulin preparations tested as antigens

				NaCl	Na-acetate	MPOB	ZnCl ₂
				(mg per ml)			
Species							
Neutral soluble insulin Actrapid ^R	40 iu/ml	pig		7	1.4	1	—
Insulin zinc suspension Monotard ^{R*}	40 iu/ml	pig		7	1.4	1	0.17

*Specially purified by gel filtration chromatography and anion exchange chromatography 12 iu/ml amorphous and 28 iu/ml crystalline insulin.

Table 2. PHA response in insulin-dependent diabetics (IDD) with acute insulin allergy (two) or a history of insulin allergy (13) and 15 healthy controls matched in respect of age and sex

Diabetic patients					Normal subjects			
Autol. plasma		AB serum			Autol. plasma		AB serum	
PHA		PHA			PHA		PHA	
No.	response	Control	response	Control	response	Control	response	Control
1	269,976	737	76,423	2,395	177,564	970	177,451	718
2	119,529	1,193	82,864	512	366,186	1,310	259,812	1,299
3	178,568	1,300	60,140	347	314,028	6,246	292,477	1,783
4	95,006	259	131,994	603	310,167	3,053	212,669	3,590
5	122,319	2,957	77,240	2,664	292,558	588	210,698	378
6	173,108	2,467	189,448	2,591	105,495	611	140,683	410
7	36,130	1,630	124,988	1,100	250,884	987	226,718	365
8	96,758	9,332	57,459	1,742	169,690	2,080	120,668	641
9	164,272	1,630	168,523	756	287,086	3,741	319,337	5,411
10	305,460	3,485	248,232	6,850	126,807	841	133,629	147
11	238,092	566	240,469	487	241,541	1,059	178,410	1,038
12	146,257	1,476	174,816	3,271	248,940	1,579	291,764	9,738
13	395,775	9,243	418,133	3,426	80,991	1,021	138,945	805
14	419,438	4,630	481,179	1,681	112,607	589	114,871	570
15	317,804	8,648	339,760	5,724	313,459	1,471	258,462	1,902

All results are in c.p.m.

20% autologous plasma the mean PHA response of IDD being $205,233 \pm 29,585$ (c.p.m. $\pm$ s.e.m.) did not differ significantly from the mean value of normal subjects, which was $226,734 \pm 23,487$. Analogous results were obtained in cultures supplemented with 20% AB serum, where the mean values were $191,445 \pm 34,155$ in IDD respectively $205,106 \pm 17,590$ in controls. At the time of the experiments all patients have been under good metabolic control.

Effect of Actrapid on lymphocyte transformation

The effect of Actrapid on lymphocyte transformation could be determined in 13 IDD with a history of insulin allergy, two IDD with acute insulin allergy, nine diabetic patients before institution of insulin therapy as well as in nine control subjects (results shown in Tables 3–5).

In eight allergic IDD (cultures supplemented with autologous plasma) respectively only three, when cultures were supplemented with AB serum, the stimulation indices were greater than the mean value of normal subjects + 2 s.d., indicating a sensitization to the drug.

Table 3. Stimulation of peripheral blood lymphocytes by insulin Actrapid and Monotard in nine normal subjects

No.	20% autol. plasma		20 % AB serum	
	Actrapid (4 iu/ml)	Monotard (4 iu/ml)	Actrapid (4 iu/ml)	Monotard (4 iu/ml)
1	7,797/0.9	50,267/6.0	9,947/0.8	20,360/1.7
2	2,139/0.9	35,389/14.9	3,792/1.3	20,974/7.2
3	7,465/0.8	—	6,811/1.0	19,272/2.7
4	2,375/0.8	—	2,533/0.8	—
	—	30,493/30.3	—	9,914/15.1
5	4,183/1.1	44,065/11.5	3,485/0.7	33,954/7.0
6	1,968/1.1	31,825/18.5	3,445/0.7	28,299/5.8
7	1,242/0.6	12,637/5.7	2,285/2.6	27,354/31.5
8	8,022/0.9	6,582/0.8	780/1.4	1,414/2.5
9	556/0.7	54,331/69.3	493/1.9	23,896/92.2

All results are in c.p.m./SI.

Table 4. Stimulation of peripheral blood lymphocytes by insulin Actrapid and Monotard in insulin-dependent diabetics (IDD) with acute insulin allergy (number 9 and 12) or a history of insulin allergy

No.	20% autol. plasma		20% AB serum	
	Actrapid (4 iu/ml)	Monotard (4 iu/ml)	Actrapid (4 iu/ml)	Monotard (4 iu/ml)
1	4,668/6.3	10,906/14.8	1,595/0.7	22,627/9.4
2	664/0.5	—	1,791/3.5	—
3	787/0.6	—	—	—
4	963/3.7	—	901/1.5	—
5	10,480/3.5	3,271/1.1	7,487/2.8	7,825/3.0
6	2,077/0.8	3,274/1.3	2,525/1.0	8,280/3.2
7	1,610/1.0	—	1,329/1.2	—
8	10,119/1.1	—	716/0.4	—
9	1,950/1.2	56,512/34.8	1,093/1.5	36,962/48.9
10	2,371/0.7	46,284/13.3	8,636/1.3	18,581/2.7
11	8,088/14.3	7,870/13.9	19,681/40.4	8,674/17.8
12	16,107/10.9	72,385/49.0	5,741/1.2	41,290/12.6
13	27,224/3.0	101,888/11.0	5,464/1.6	40,823/11.9
14	14,875/3.2	97,152/21.0	1,181/0.7	39,301/23.3
15	12,195/1.4	168,338/19.5	10,488/1.8	62,253/10.9

All results are in c.p.m./SI.

The mean stimulation indices in cultures supplemented with 20% autologous plasma were 3.48 ± 1.05 ($\pm$ s.e.m.) in allergic IDD with a range from 0.5 to 14.3, 1.31 ± 0.23 in diabetics before insulin therapy (range: 0.7–2.6) and 0.86 ± 0.06 in normal subjects (range: 0.6–1.1).

In cultures supplemented with 20% AB serum the mean stimulation indices were 4.26 ± 2.78 in allergic IDD (range: 0.4–40.4), 1.14 ± 0.71 in diabetics before insulin therapy (range: 0.2–2.4) and 1.24 ± 0.22 in normal subjects (range: 0.7–2.6).

Only in the experiment set up with addition of autologous plasma the difference between normal

Table 5. Stimulation of peripheral blood lymphocytes by insulin Actrapid and Monotard in diabetic patients never treated with insulin injections (nine cases, where insulin therapy was subsequently initiated)

No.	20% autol. plasma		20% AB serum	
	Actrapid (4 iu/ml)	Monotard (4 iu/ml)	Actrapid (4 iu/ml)	Monotard (4 iu/ml)
1	36,703/2.6	93,852/6.7	16,269/2.4	42,588/6.4
2	567/1.1	6,469/12.3	1,723/1.5	10,442/9.1
3	—	—	2,821/1.9	14,926/9.9
4	150/0.7	497/2.5	249/0.9	3,246/11.6
5	2,536/1.6	15,468/10.1	1,691/0.9	13,240/6.9
6	2,563/1.0	15,920/6.5	799/0.5	6,281/3.7
7	512/0.7	15,148/20.1	220/0.2	9,698/10.9
8	14,037/1.8	44,753/5.9	2,888/0.6	15,679/3.3
9	5,048/1.0	48,997/9.6	6,150/1.4	42,627/9.7

All results are in c.p.m./SI.

subjects and allergic IDD in mean stimulation of lymphocytes was found statistically significant ($P < 0.05$).

Effect of Monotard on lymphocyte transformation

Analogous experiments have been performed with Monotard (results shown in Tables 3–5).

Monotard induced a marked ^3H -thymidine uptake both in diabetic and in normal subjects with stimulation indices of up to 92.2. Surprisingly, the mean stimulation index was even higher in normal subjects than in diabetic patients being 19.6 ± 7.8 ($\pm$ s.e.m.) in normal subjects, 18.0 ± 4.6 in allergic IDD and 9.2 ± 1.9 in diabetics before insulin therapy, when cultures were performed in 20% autologous plasma. Similar results were obtained in cultures supplemented with 20% AB serum. In virtually no diabetic patient was the stimulation index greater than the mean value of normal subjects + 2 s.d., although the maximal stimulation indices went up to 48.9 and 49.0.

Regression analysis of the results in normal subjects showed a positive correlation ($r = 0.966$ for autologous plasma and $r = 0.976$ for AB serum) between the PHA response and the stimulation by Monotard (see Fig. 1). Very wide individual variations in stimulation indices were found with Monotard (range: 0.8–92.2) as well as with PHA (range: 8.1–800).

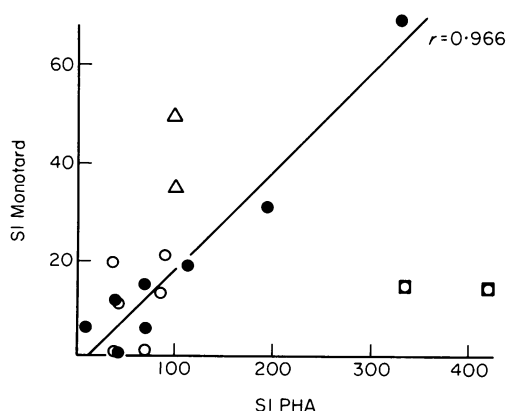


Fig. 1. Correlation between PHA response and stimulation by insulin Monotard. Results of cultures performed with addition of 20% autologous plasma. Normal subjects (●), diabetic patients with a history of insulin allergy (○), diabetic patients with acute insulin allergy (Δ), diabetic patients with moderately elevated insulin antibody titres (□).

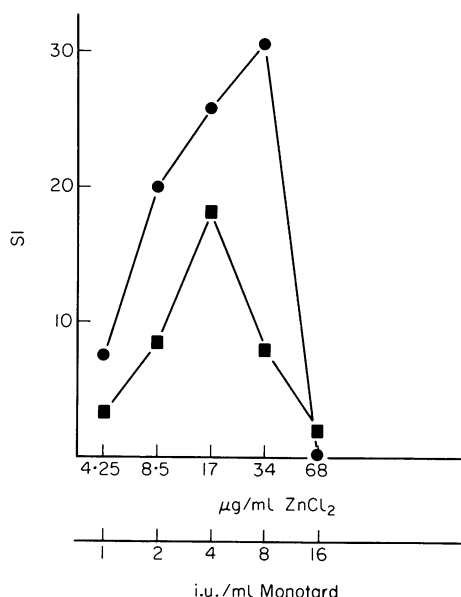


Fig. 2. Dose-response curves of stimulations obtained by ZnCl_2 (●—●) and insulin Monotard (■—■). Cultures were performed with addition of 20% autologous plasma.

Kinetics of ZnCl_2 stimulation of human lymphocytes

Since zinc is known to influence lymphocyte transformation, kinetics of the stimulation of human lymphocytes by ZnCl_2 and Monotard were compared. The results of cultures performed in medium supplemented with 20% autologous plasma are given in Fig. 2. The dose-response curves were obtained with lymphocytes of a normal subject and were roughly parallel. With ZnCl_2 the maximal response was found at a concentration of 34 $\mu\text{g/ml}$ ZnCl_2 corresponding to the zinc content of 8 i.u./ml Monotard, the stimulation index being 30.7. At a concentration of 17 $\mu\text{g/ml}$ ZnCl_2 the stimulation index was slightly lower (25.8). With Monotard the maximal response was obtained at a concentration of 4 i.u./ml with a stimulation index of 18.0.

In another experiment the culture was set up with addition of 34 $\mu\text{g/ml}$ ZnCl_2 and harvested daily from day 3 to 6. The maximal uptake of ^3H -thymidine was found at day 4 (results not shown).

DISCUSSION

Conflicting results considering the PHA response of peripheral blood lymphocytes in diabetic patients have been reported so far. Our data established in IDD indicate that non-specific transformation by PHA is not impaired in comparison to normal subjects matched in respect of age and sex. This is in conformity with MacCuish *et al.*, (1974) who did not find any difference in PHA response of lymphocytes between well controlled IDD and matched controls either, but noticed, that the PHA response was significantly impaired in poorly controlled diabetics. Our results, however, differ from those of Delespesse *et al.* (1974), who found a depressed PHA response in both IDD and non-insulin-dependent diabetics. Variation in age and sex distribution as well as in metabolic control may account for differences between the several studies. Furthermore, the PHA concentration used might be critical.

Lymphocyte transformation in presence of an antigen is a widely used *in vitro* test of cellular immunity. So far various investigators (Halpern *et al.*, 1967; Federlin, Kriegbaum & Flad, 1968; Federlin, 1971; Feinglos & Jegasothy, 1979; Diem & Teuscher, 1979) have shown that insulin of porcine or bovine origin can induce lymphocyte transformation in lymphocytes from diabetic patients with both immediate or delayed type hypersensitivity to commercial insulin.

The results of lymphocyte transformation tests with Actrapid were as expected. A significant stimulation ($SI > \text{mean } SI \text{ of normal subjects} + 2 \text{ s.d.}$) was detectable in eight allergic patients out of 15 (autologous plasma) respectively three out of 14 (AB serum) as well as in three out of eight diabetics before insulin therapy (autologous plasma). Insulin induced lymphocyte transformation in IDD without any clinical sign of insulin allergy as well as in newly diagnosed diabetics has been reported by MacCuish *et al.* (1975). These experiments, however, were done with insulin preparations containing a considerable amount of zinc.

In our culture conditions, commercial insulin-zinc suspensions, Lente (results not shown) and Monotard, induced a marked lymphocyte transformation even in normal subjects. No significant difference in stimulation of lymphocytes by insulin zinc suspensions between normal subjects, IDD with a history of insulin allergy or acute insulin allergy and diabetics tested prior to insulin treatment could be established. Consistently, Monotard led to increased stimulation indices, yet with a wide individual variation. Most probably, this marked lymphocyte stimulation is non-specific and due to the zinc content of these preparations. Zinc is inducing lymphocyte transformation in serum containing media (Kirchner & Rühl, 1970; Loeb, 1974) and it plays a major role in PHA induced lymphocyte transformation (Phillips & Azary, 1973; Williams & Loeb, 1973). Dose-response analysis showed roughly parallel curves for Monotard and ZnCl_2 solutions with the respective amount of Zn^{++} , although the general response to Monotard was slightly lower. The maximal ^3H -thymidine uptake in cultures set up with addition of ZnCl_2 was found on day 4. Non-specific mitogen induced lymphocyte transformation normally reaches its maximum on day 3 or 4, whereas specific antigen induced transformation has a maximum on day 6. These findings suggest that Monotard had to be considered as a potent, non-specific mitogen, *in vitro*, most probably due to its zinc content.

Interestingly, in normal subjects a close correlation between the stimulation indices of Monotard and PHA was found ($r=0.966$ respectively $r=0.976$), suggesting that these stimulations depend on a common parameter namely the reactivity to mitogens PHA and Zn^{++} respectively. Among the 10 allergic IDD tested with Monotard, in six a similar relation was found. The two patients with acute insulin allergy (patients No. 9 and 12) had a relatively high, while two patients with moderately elevated insulin antibodies (insulin binding capacity of serum, patients No. 1 and 11) had a relatively low stimulation with Monotard.

Further studies will be required to confirm this correlation. Nevertheless, some conclusions are already possible from these observations. (1) Lymphocyte transformation tests can provide only a weak argument in proof of insulin allergy due to zinc insulin or zinc, which has recently been claimed (Feinglos & Jegasothy, 1979), since so many normal subjects also show stimulation. (2) It is generally accepted that multiple doses of insulin solutions are less immunogenic than treatment with insulin zinc suspensions. This effect may be due to the zinc content of these preparations and not only to their special pharmacokinetic properties. (3) Immune responses and *in vitro* reactions in IDD and untreated diabetics have to be interpreted very carefully: they may be due to allergic phenomena as well as to non-specific effects of zinc or actually reflect mechanisms relevant in the pathogenesis of diabetes mellitus.

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