

Cytokine overproduction in healthy first degree relatives of patients with IDDM

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Summary Healthy family members of patients with insulin-dependent diabetes mellitus (IDDM) are known to share a number of immunological abnormalities with their affected relatives. Since monocyte and type 1 T-cell-derived cytokines contribute to the pathogenesis of IDDM, we studied the production of these cytokines in the healthy first degree relatives of 29 children with IDDM. We report that circulating tumour necrosis factor- α (TNF- α) and soluble interleukin-2 (sIL-2) receptor were present in increased amounts in non-diabetic family members at levels similar to those found in the diabetic children (duration of disease 3 months–5 years). Furthermore,

marked hypersecretion of IL-1 α and TNF- α by mitogen-stimulated peripheral blood mononuclear cells was found in both diabetic and healthy family members. Abnormalities of cytokine production in healthy relatives did not correlate with the presence of islet cell antibodies or with HLA DR type. These data indicate that healthy family members of patients with IDDM exhibit overproduction of a number of cytokines that have been implicated in diabetogenesis. [Diabetologia (1998) 41: 343–349]

Keywords Insulin-dependent diabetes mellitus, first degree relatives of diabetic patients, cytokines.

Insulin-dependent diabetes (IDDM) is believed to result from autoimmune destruction of the beta cells of the pancreas [1–3]. A mononuclear cell infiltrate (insulitis), consisting primarily of T cells and macrophages, is characteristically found in the islets of Langerhans both at diagnosis [1] and following recurrence of disease in pancreatic iso-grafts [2, 3].

There is increasing evidence that cytokine production plays a key role in the pathogenesis of a number of autoimmune disorders including IDDM [4].

Monocyte-derived cytokines, including interleukin (IL)-1 β and tumour necrosis factor- α (TNF- α), are cytotoxic to beta cells and also inhibit insulin secretion in vitro [5, 6]. Production of interferon- γ (IFN- γ) by T cells may also promote immune targeting of beta cells as a result of increased expression of HLA class I, HLA class II, adhesion molecules [7] and Fas [8] by pancreatic islets. Furthermore, IFN- γ is directly cytotoxic to islet cells [5, 9] and synergistically enhances islet cell destruction by TNF and IL-1 [9].

We have previously reported that levels of circulating monocyte (IL-1 α , TNF- α) and T cell type 1 subset cytokines (IL-2, IFN- γ) are elevated in patients at the time of diagnosis of IDDM [10], in agreement with a number of other studies [11–14]. By contrast, production of type 2 cytokines (IL-4 and IL-10) was not raised, in keeping with the proposal that a T helper 1 dominant immune response plays a central role in disease pathogenesis [15, 10]. Because of obvious difficulty in obtaining tissue samples, study of local cytokine production in human pancreata has been limited [16, 17]. We have demonstrated, how-

Received: 26 August 1997 and in revised form: 24 October 1997

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Abbreviations: TNF- α , Tumour necrosis factor- α ; IL, interleukin; IFN- γ , interferon- γ ; LPS, lipopolysaccharide; sIL-2r, soluble IL-2 receptor; ELISA, enzyme-linked immunosorbent assay; ICA, islet cell antibody; IDDM, insulin-dependent diabetes mellitus; PHA, phytohaemagglutinin; PBMC, peripheral blood mononuclear cells; CTLL, cytotoxic T lymphocyte line

ever, a strong correlation between systemic and intra-islet cytokine profiles in the NOD mouse model of spontaneous IDDM [18]. It is therefore conceivable that abnormalities in circulating cytokine levels also reflect pancreatic events in man and may be of direct relevance to diabetogenesis.

One approach to investigating the immunopathogenesis of diabetes is to study first degree relatives of affected individuals. An important advantage of such studies is that they eliminate the confounding effects of chronic hyperglycaemia and insulin therapy upon the immune system [19, 20]. Although at relatively low risk of progression to IDDM, these individuals exhibit an increased prevalence of both autoantibodies and T cells reactive against islet cell autoantigens [21–24]. In addition, we have demonstrated a range of lymphocyte subset abnormalities in peripheral blood of healthy siblings and parents of diabetic children [25]. In the present study we have extended these investigations by measuring circulating and mitogen-stimulated cytokine production in a series of diabetic children and their non-diabetic parents and siblings. Since much of the genetic susceptibility to IDDM maps to the MHC class II region, we have also studied the association between cytokine levels in these individuals and HLA DR types.

Subjects and methods

Diabetic patients and family members. We have previously studied 31 families, all with a single index case of childhood IDDM [fully described in 25]. In the present study, samples from 29 of these families were analysed for cytokine production as described below. Ethical permission for this study was obtained from the Farnborough Hospital ethics committee and all participants gave their informed consent. In all families, samples were obtained from the affected child. In 8 families, samples were obtained from both parents and 2 siblings; in two families from both parents only; in the remainder, samples were obtained from the mother and 3 siblings ($n = 1$), 2 siblings ($n = 1$), one sibling ($n = 1$) or no siblings ($n = 3$); in one family, samples were obtained from the father and index case only.

There was a total of 29 children with IDDM (14 males, mean age \pm SD, 10.8 ± 4.5 years). Duration of diabetes in affected children ranged from 3 months to 5 years ($n = 29$, mean \pm SD; 2.46 ± 1.19 years; median = 2.25 years). IDDM was defined in accordance with the guidelines of the National Diabetes Data Group [26]. In addition, we studied 51 parents (25 males, age 39.1 ± 7.0 years) and 34 siblings (15 males, mean age 12.8 ± 4.3 years) none of whom had diabetes. As control subjects, blood was obtained from 25 healthy adults (11 males, age 35.4 ± 6.6 years) recruited from the staff of King's College Hospital and 25 healthy children (12 males, age 11.4 ± 3.8 years) who were recruited by the Medway and Gillingham Branch (Kent) of the Children's Liver Disease Foundation. None of the control subjects had a family history of IDDM. Blood was drawn from control subjects during the same period and at the same time of day as for the study group to avoid biases resulting from seasonal, diurnal or technical variations. Samples were only obtained from individuals with-

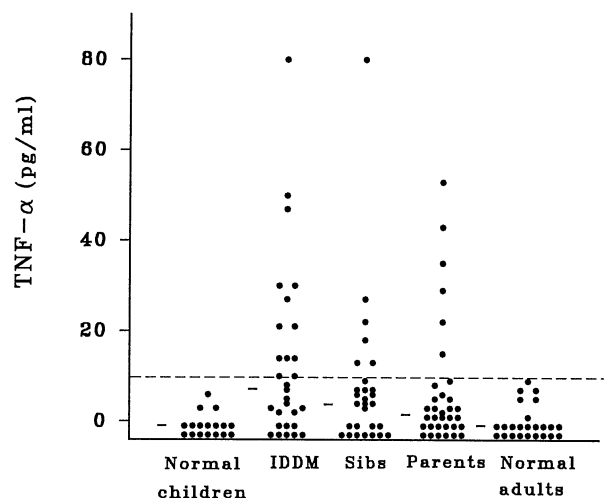


Fig. 1. Circulating TNF- α values in the serum of children with IDDM, in their parents, siblings and control subjects. The dashed line represents the upper limit of the normal control values. Levels of TNF- α were significantly higher in children with IDDM, in their siblings and parents when compared with normal control subjects ($p < 0.02$). Horizontal bars represent median values

out symptoms or clinical evidence of infection. To allow for the effects of a chronic autoimmune disease, 21 patients were studied with Graves' disease (GD) but without diabetes and seen consecutively at the Endocrine Clinic at King's College Hospital (9 males; mean age 40 ± 9.1 years).

Blood was taken and allowed to clot for 30 min at room temperature. Serum was separated from clotted blood by centrifugation at 1500g, aliquoted and stored at -70°C . In some cases (especially in children), measurement of cytokines was incomplete due to limited availability of serum.

Mitogen-induced secretion of cytokines. Peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood by Ficoll-Hypaque density gradient centrifugation. Cell viability was over 90 % as assessed by trypan-blue exclusion. Cells were then divided into three tubes each containing 10^6 cells/ml in RPMI \pm 10 % fetal calf serum. The first and second were treated with 1 $\mu\text{g}/\text{ml}$ of phytohaemagglutinin (PHA – preferentially stimulates T cells) or lipopolysaccharide (LPS – preferentially stimulates macrophages) respectively, while mitogen was not added to the third tube (control). After 24 h of culture at 37°C in a humidified 95 % air/5 % CO_2 incubator, supernatants were collected by centrifugation, filter sterilized (0.22 mm pore size) and stored at -20°C until tested.

Measurement of cytokines and soluble IL-2 receptor (sIL-2r). IL-2 levels were measured in PHA-stimulated and unstimulated peripheral blood mononuclear cells (PBMC) supernatants using a bioassay based upon the IL-2-dependent CTLL-16 cell line as described [10]. Levels of IFN- γ , IL-1 α , TNF- α , IL-4 and sIL-2r were measured in serum and PBMC supernatants using a sandwich enzyme-linked immunosorbent assay (ELISA) [10, 27]. IFN- γ and sIL-2r were measured using monoclonal anti-IFN- γ or anti-sIL-2r respectively according to published techniques [28, 29]. TNF- α , IL-1 α and IL-4 were measured as previously described [10]. The inter- and intra-assay coefficient of variation of these assays never exceeded 8 and 5 %, respectively. The sensitivity of the assays was 5 pg/ml (TNF- α), 10 pg/ml (IL-1 α and IL-4), 25 pg/ml (IFN- γ) and 25 U/ml (sIL-2r). When optical density values were below the

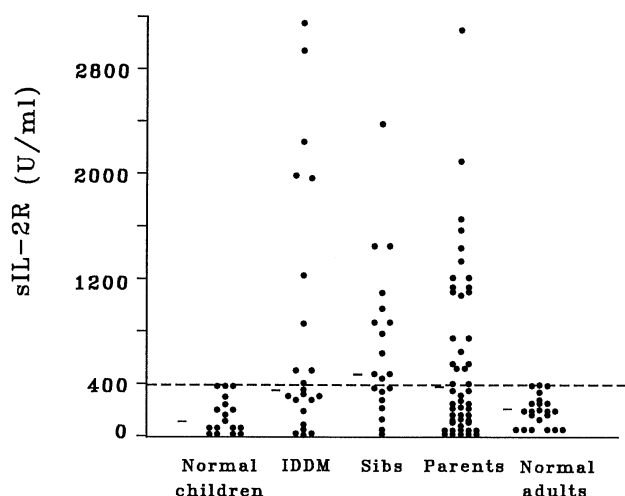


Fig. 2. Circulating sIL-2r levels in the serum of children with IDDM, in their parents, siblings and control subjects. Levels of sIL-2r were significantly higher in the diabetic children with IDDM, in their siblings and parents when compared with normal control subjects ($p < 0.001$). Horizontal bars represent median values

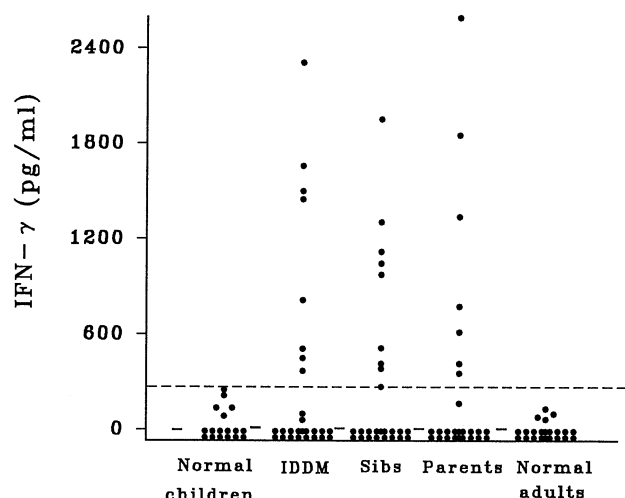


Fig. 3. Serum IFN- γ levels in children with IDDM, in their parents, siblings and control subjects. Higher levels of IFN- γ were found in the diabetic children and their family members when compared to the normal control groups, although this difference did not reach statistical significance. Horizontal bars represent median values

lowest value of the reference curve, a value of 0 pg/ml was assigned. Since non-parametric statistical comparisons were used throughout, this did not interfere with the statistical analysis.

Autoantibodies and HLA DR typing. Antibodies to islet cells (ICA) were detected by indirect immunofluorescence microscopy [25, 30]. ICA levels are presented in Juvenile Diabetes Foundation (JDF) units based on titration of the JDF standard serum on the one single pancreas used throughout the study. A sample was considered ICA-positive at a score of 5 JDF units or more. HLA DR typing was carried out as previously described [25].

Table 1. Levels of spontaneous and LPS-stimulated secretion of TNF- α by PBMC in diabetic children, their siblings and parents

Group	No	Spontaneous	With LPS
		TNF- α (pg/ml) Median (range)	TNF- α (pg/ml) Median (range)
Normal control subjects	39	47 (0–52)	372 (138–701)
Diabetic children	19	22 (0–544)	2279 (54–3761) ^a
Siblings	14	57 (0–734)	1385 (165–2134) ^a
Parents	23	80 (9–642)	2205 (605–3483) ^a

^a $p < 0.01$ compared with normal control subjects using Wilcoxon's rank sum test

Table 2. Levels of spontaneous and LPS-stimulated secretion of IL-1 α by PBMC and after LPS stimulation in diabetic children, their siblings and parents

Groups	No.	Spontaneous	With LPS
		IL-1 α (pg/ml) Median (Range)	IL-1 α (pg/ml) Median (Range)
Normal control subjects	39	0 (0–12)	82 (23–509)
Diabetic children	19	0 (0–292) ^a	195 (24–741) ^a
Siblings	14	23 (0–283) ^a	341 (110–915) ^a
Parents	23	0 (0–190) ^a	295 (0–626) ^a

^a $p < 0.01$ compared with normal control subjects using Wilcoxon's rank sum test

Statistical analysis. The distribution of the levels of TNF- α , IL-1 α , IFN- γ , IL-4 and sIL-2r did not satisfy the hypothesis of normality where this was assessed using the Kolmogorov-Smirnov goodness of fit test and levels were therefore compared using the non-parametric Wilcoxon rank sum test. Regression analysis was performed using Spearman's rank correlation. Median (range) levels of all cytokines and sIL-2r were similar in the adult control subjects and normal children. For ease of graphic presentation, upper limits of normal in Figures 1–5 are calculated after combining the control groups.

Results

Circulating cytokine and sIL-2r levels. Levels of serum TNF- α were significantly increased not only in children with IDDM but also in their siblings and parents compared to the appropriate control group ($p < 0.02$ for all) (Fig. 1). Levels of TNF- α exceeding the highest normal value (> 9 pg/ml) were found in 13/29 (45 %) of diabetic children, 6/28 (21 %) of their siblings and 6/33 (18 %) of their parents. In contrast, levels of IL-1 α were similar in diabetic children, family members and normal control subjects.

Levels of sIL-2r (a marker of T cell activation) were significantly increased in diabetic children and in their siblings and parents when compared to nor-

mal control subjects ($p < 0.001$ for all) (Fig. 2). Ten of 22 (46 %) diabetic children, 11/20 (55 %) of their siblings and 20/50 (40 %) of their parents had sIL-2r levels exceeding the highest normal value (> 400 U/ml).

We next measured circulating levels of IFN- γ and IL-4, which are representative of type 1 and type 2 T-cell cytokines, respectively. Levels of IFN- γ exceeding the highest normal value (> 250 pg/ml) were found in 8/27 (30 %) diabetic children, 8/26 (31 %) of their siblings and 7/26 (27 %) of their parents ($p < 0.23$; 0.20 and 0.14, respectively, Fig. 3). Levels of IL-4 exceeding the highest normal value (> 85 pg/ml) were found in 2/13 (15 %) diabetic children, 2/15 (13 %) of their siblings and 2/15 (13 %) of their parents.

No correlation was found between levels of TNF- α , IL-1 α , IFN- γ and IL-4 in diabetic children, their siblings or their parents. However, 14/29 (48 %) of diabetic children, 12/34 (35 %) of their siblings and 12/51 (24 %) of their parents showed elevated levels of more than one cytokine and sIL-2r.

Spontaneous and mitogen-stimulated cytokine production by PBMCs

TNF- α secretion. Levels of spontaneous TNF- α secretion were similar in diabetic children, their siblings and their parents when compared with normal control subjects (Table 1). By contrast, LPS-stimulated TNF- α production was elevated in diabetic children and in their siblings and parents when compared with normal control subjects (Table 1).

IL-1 α secretion. When compared to normal control subjects, levels of spontaneous IL-1 α secretion by PBMC were increased in diabetic children and in their siblings and parents. A similar pattern of increase was also observed after LPS stimulation in all of these groups, compared to normal control subjects (Table 2).

Secretion of T-cell cytokines. Levels of PHA-stimulated secretion of IL-2 (calculated as the percentage of maximal CTLL-16 stimulation), IFN- γ and IL-4 by PBMC were similar in diabetic children, their siblings and parents and normal control subjects (Tables 3–5).

Association between cytokine levels and ICA. ICA were detected in 18 diabetic children (62 %), 4 siblings (12 %) and 2 parents (4 %). Duration of disease was shorter in diabetic children who were positive (2.06 ± 1.09 years) compared to those who were negative for ICA (3.08 ± 1.11 years; $p < 0.02$). Diabetic children with ICA had higher levels of IFN- γ compared with their ICA-negative counterparts (median 100 pg/ml; range 0–2310 vs 0 pg/ml, 0–615; $p < 0.05$). A similar trend for sIL-2r being higher in ICA positive diabetic children was seen, but failed to reach

Table 3. Levels of spontaneous and PHA-stimulated secretion of IL-2 by PBMC in diabetic children, their siblings and parents

Groups	No.	Spontaneous	With PHA
		IL-2 (% of CTLL stimulation) Median (range)	IL-2 (% of CTLL stimulation) Median (range)
Normal control subjects	39	0 (0–0)	32 (11–64)
Diabetic children	19	0 (0–0)	35 (20–59)
Siblings	14	0 (0–0)	134 (9–38)
Parents	23	0 (0–0)	28 (9–45)

No significant difference compared with normal control subjects using Wilcoxon's rank sum test

Table 4. Levels of spontaneous and PHA stimulated secretion of IFN- γ by PBMC in diabetic children, their siblings and parents

Groups	No.	Spontaneous	With PHA
		IFN- γ (pg/ml) Median (range)	IFN- γ (pg/ml) Median (range)
Normal control subjects	39	0 (0–0)	0 (0–246)
Diabetic children	19	0 (0–0)	51 (0–1200)
Siblings	14	0 (0–0)	71 (0–950)
Parents	23	0 (0–0)	0 (0–650)

No significant difference compared with normal control subjects using Wilcoxon's rank sum test

Table 5. Levels of spontaneous and PHA stimulated secretion of IL-4 by PBMC in diabetic children, their siblings and parents

Groups	No.	Spontaneous	With PHA
		IL-4 (pg/ml) Median (range)	IL-4 (pg/ml) Median (range)
Normal control subjects	19	0 (0–0)	80 (45–125)
Diabetic children	11	0 (0–0)	68 (42–634)
Siblings	14	0 (0–0)	73 (54–132)
Parents	23	0 (0–0)	95 (68–362)

No significant difference compared with normal control subjects using Wilcoxon's rank sum test

conventional levels of statistical significance ($p = 0.08$). Increased levels of TNF- α , IL-1 α or IL-4 associated with positivity for ICA were not seen in any of the groups analysed. There was no relation between concentrations of any of the cytokines tested and levels of ICA.

Association between cytokine levels and HLA DR. The diabetic children, siblings and parents examined

in the present study have all previously been fully typed for HLA DR [25]. All individuals were assigned to one of the following four groups: 1) homozygous DR3/3 or heterozygous DR3/X; 2) heterozygous DR3/DR4; 3) homozygous DR4/4 or heterozygous DR4/X and 4) DRX/X (where X is not DR3 or 4). When categorized in this manner, there was no significant effect of HLA DR haplotype upon circulating or mitogen-stimulated cytokine levels in any of the three groups examined here (data not shown).

Association between cytokine levels, metabolic control and duration of diabetes. Among the diabetic children, there was no relationship between the increased levels of cytokines and random blood glucose, haemoglobin A_{1c}, or duration of IDDM (data not shown).

Discussion

The major finding of the current study is that monocyte and T-cell activation is found in healthy first degree relatives of children with IDDM. Thus, increased circulating levels of TNF- α and sIL-2r are present in non-diabetic family members (i.e., siblings and parents), as well as in their affected relatives. In addition, marked hypersecretion of IL-1 α and TNF- α by mitogen-stimulated PBMC was found in both diabetic children and healthy relatives. These data clearly indicate that augmented cytokine production is prevalent in healthy family members of diabetic patients.

The findings described in our study present an intriguing paradox. Despite the abnormalities of cytokine production demonstrated here, most of the healthy family members studied will never develop diabetes. How can this fact be reconciled with the wealth of evidence implicating both monocyte and type 1 T-cell cytokines in the pathogenesis of IDDM [31, 32]? The most likely explanation for this relates to the absolute levels at which these cytokines are produced *in vivo*. We have previously shown that circulating levels of IL-2, IFN- γ , TNF- α and IL-1 α are considerably higher at the time of diagnosis of diabetes and in the prediabetic period than those reported here in healthy family members or in patients with diabetes of 3 months to 5 years duration [10]. Furthermore, a clear bias toward activation of type 1 T cells is not apparent in the family members studied, in contrast to the findings observed in recent onset diabetic patients [10]. Thus, it appears that while the family members share an increased activation of monocytes and T cells with their diabetic relatives, this response never reaches the peak observed in the prediabetic/early diabetic period. An alternative possibility is that the potential diabetogenic effects of these cyto-

kines may be countered in healthy family members by production of cytokine antagonists [33].

We found no association between circulating or mitogen-stimulated cytokine production and positivity for ICA in healthy family members. ICA represent a risk factor for progression to IDDM when found in relatives of affected patients [34]. A number of other autoantibodies have also been associated with increased risk of diabetes in first degree relatives including those directed to glutamic acid decarboxylase (GAD), IA-2 and insulin [35]. Consequently, it will be interesting to determine in future studies whether cytokine levels correlate with the presence of any of these markers of islet cell autoimmunity.

In contrast to the healthy family members, we found that the presence of ICA in the diabetic children was associated with increased levels of IFN- γ . Since we have shown that positivity for ICA and elevated cytokine levels are most marked in the early stages of diabetes [10], this association most likely reflects a relatively shorter duration of disease in these patients.

Why do diabetic patients and their family members exhibit such a spectrum of immunological abnormalities? One possible explanation is that of a heightened immune responsiveness in these individuals [25]. This hypothesis is strongly supported by the markedly enhanced output of monocytic cytokines by PBMCs from these individuals following stimulation with LPS. It has previously been shown that overactivity of the immune system represents a possible predisposing factor to autoimmune disease in individuals with particular HLA class II alleles (HLA DR3/DR4) [36]. In the present study, we HLA DR typed all of our subjects and found no association between DR3 or 4 and either circulating levels of cytokines or their secretion by mitogen-stimulated PBMC. This suggests that these "diabetogenic" alleles do not exert a major influence upon cytokine production in diabetic children or in their relatives. This finding is in agreement with the study of Mølviq et al. [37] who did not find a significant association between production of IL-1 β and TNF- α by monocytes and HLA DR phenotype.

It is possible that other alleles are linked to the abnormalities in cytokine secretion reported here. Polymorphisms have been described in the TNF- α [38–40], IFN- γ [7] and IL-1 β [41] genes in diabetic patients, some of which have been shown to influence the level of expression of the corresponding cytokine. The case of TNF- α is particularly interesting since there is evidence that this cytokine regulates production of other soluble factors including IL-1 [4]. Furthermore, the TNF- α gene is located on chromosome 6 in close proximity to the MHC class II region. Thus, future studies must address the question as to whether there is an association between TNF- α gene polymorphisms and the abnormalities of cytokine production, as observed in the present paper.

In summary, we have demonstrated that healthy family members of children with IDDM exhibit overproduction of a range of cytokines which have been implicated in the pathogenesis of this disorder. Together with our previous findings [10, 25], we have clearly shown that many of the immunoregulatory abnormalities involved in diabetogenesis are shared by healthy family members. The focus of current research must be to determine what factors are required, in addition to this background, to tip the balance towards beta-cell destruction.

Acknowledgements. We thank Dr. R.Thorpe (National Institute for Biological Standards and Controls, Mill Hill, UK) and Dr. D.L.Nelson (National Institute of Health, Bethesda, USA) for anti-IL-1 α and anti-sIL-2r antibodies, respectively.

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