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Evidence that defective gamma interferon production in patients with primary immunodeficiencies is due to intrinsic incompetence of lymphocytes

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SUMMARY

We have selected 11 patients with primary immunodeficiency disorders predominantly affecting T lymphocyte function (four with ataxia-telangiectasia (AT), four with common variable immunodeficiency (CVI) and one each with Wiskott–Aldrich syndrome, hyper-IgE syndrome and combined immunodeficiency) with defective gamma interferon (IFN-γ) production *in vitro*. Induction with phytohaemagglutinin showed low interleukin 2 (IL-2) production concomitant with reduced IFN-γ titres. However the addition of 10 U/ml of rIL-2 to cultures stimulated with staphylococcal enterotoxin B or galactose oxidase failed to restore IFN-γ production in defective cases. IFN-γ was titrated by both bioassay and immunoradiometric assay, ruling out the possible release of inactive or altered IFN-γ molecules. Normal levels of IFN-γ were found in parents of patients with AT, as well as in two AT and two CVI cases, demonstrating heterogeneity of defects within these syndromes. Soluble inhibitors or cellular suppression of IFN-γ were not observed in mixing experiments. The possibility that defective interaction between accessory cells and T lymphocytes might account for the poor response to the inducing agents was ruled out as no IFN-γ was produced using a calcium ionophore—which bypasses this step—in seven patients with absolute IFN-γ deficiency.

Keywords gamma interferon immunodeficiency interleukin 2

INTRODUCTION

Patients with primary immunodeficiency diseases (ID) suffer from inherited, congenital or acquired disorders of either humoral or cell-mediated immunity (Rosen, Wedgwood & Eibl, 1986). The resulting susceptibility to microbial infections may be due to the lack of specific components of the immune system, or to disability of the compensatory mechanism of non specific immunity or a combination of both. In several ID, including combined immunodeficiencies (CID), ataxia-telangectasia (AT), Wiskott–Aldrich syndrome (WA) and common variable immunodeficiency (CVI), a functional depression of T lymphocyte functions is commonly observed (Rosen *et al.*, 1986). T lymphocytes regulate the function of other cell types with the production of potent immunoregulatory molecules (lymphokines) including interleukin-2 (IL-2) and gamma interferon (IFN-γ). The effect of lymphokines on the immune system is complex, since their action is exerted on different cell types, and mediated by the secretion of other molecules. Interleukin 2 has been shown to induce and increase IFN-γ production (Farrar, Johnson & Farrar, 1981; Kasahara *et al.*, 1983; Reem & Ning-Hsing, 1984) and both B and T lymphocytes express receptors for IL-2 (Greene & Leonard, 1986); IFN-γ has also been reported to enhance IL-2 production (Frasca *et al.*, 1985).

We and others have reported deficiencies of IFN-γ production in patients with ID predominantly affecting T lymphocyte function (Virelizier *et al.*, 1979; Matricardi *et al.*, 1984; Paganelli *et al.*, 1984). In most of these syndromes, a defective production of IL-2 has also been observed (Lopez-Botet *et al.*, 1982; Paganelli *et al.*, 1983; Kruger *et al.*, 1984). However the relationship of these defects, and the level of the defective step have not been investigated.

Here we report a study of lymphokine production in a selected group of ID patients, previously screened for defective IFN-γ *in vitro* synthesis, showing that the addition of IL-2 does not restore IFN-γ release, and that several inducing agents, including calcium ionophore, are unable to bypass the defective step, suggesting an intrinsic cause for the inability to produce IFN-γ.

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MATERIALS AND METHODS

Patients and controls

Eleven patients with various primary ID were selected: four had ataxia-telangiectasia (AT), four had common variable immunodeficiency (CVI), and one each had hyper-IgE syndrome (HIgE), combined immunodeficiency (CID) with hyper-IgM (Fiorilli *et al.*, 1986) and Wiskott–Aldrich (WA) syndrome. Most of them had been previously tested at least once for IFN-γ production and were found to produce abnormally low amounts of this lymphokine. The others belonged to disease groups where defective IFN-γ has been described (Virelizier *et al.*, 1979; Matricardi *et al.*, 1984; Paganelli *et al.*, 1984). Eleven young healthy volunteers were also studied as normal controls. Five parents of AT patients were investigated in order to detect the association of cell mediated immune defects with the genetic transmission of the disease.

Mononuclear cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood samples by Ficoll-Isopaque density gradient centrifugation. After three washes in RPMI-1640 containing 10% fetal calf serum (Gibco, Grand Island, NY), PBMC were checked for viability (always >90%), counted and resuspended in the same culture medium.

PBMC stimulation in vitro

Cells (10^6 in 1 ml of complete medium) were cultured for 24 h in the presence or absence of optimal stimulating concentration of phytohaemagglutinin (PHA-P, Wellcome) at 37°C in a humidified 5% CO_2 atmosphere. The supernatants were then collected under sterile conditions and stored at −20°C until analysis for IL-2 and IFN-γ titration. For IFN-γ we also used the following inducers: staphylococcal enterotoxin B subunit (SEB) at 0·2 μg/ml for 10^6 cells, with culture conditions as for PHA; treatment with the enzyme galactose oxidase (GO, Worthington Biochem. Co., Freehold, NJ) at 10 U/ml for 10^7 PBMC for 30 min at 24°C as previously detailed (Dianzani *et al.*, 1979); calcium ionophore A23187 (Sigma, St Louis, MO) 10 μM for 10^6 PBMC. Supernatants were collected after 18–20 h of culture at 37°C and stored at −20°C. Recombinant IL-2 (rIL-2, Biogen, Geneva, Switzerland) at 10 U/ml was added to unstimulated and SEB-induced or GO-induced PBMC at the beginning of the cultures, and supernatants collected as described. Co-cultures of PBMC from a normal subject and two ID patients were set up with 5×10^5 PBMC from each partner in 1 ml in presence of SEB or GO.

IL-2 titration

Interleukin 2 in the supernatants was measured by its ability to sustain the growth of the IL-2-dependent murine T cell line HT2 (Kappler *et al.*, 1981; a gift from Dr O. Pontesilli, Denver). Different dilutions of cell supernatants were plated in duplicate with 4000 HT2 cells in a total volume of 200 μl in plastic 96-well microculture plates (Linbro). Both a reference standard derived from the Jurkat cell line, and rIL-2 were used in each assay to normalize the results.

After 24 hours of culture each well was pulsed for 16 h with 0·5 μCi of ^{3}H-thymidine (Amersham) and harvested on fibreglass paper. The incorporated thymidine was determined by liquid scintillation counting (Packard Instr., Warrenville, IL) and IL-2 expressed with reference to standards as U/ml by probit analysis according to the method of Gillis *et al.* (1978).

Table 1. Results of IL-2 and IFN-γ determinations by bioassays after *in vitro* stimulations of PBMC with PHA

No	Diagnosis	IL-2 (U/ml)	IFN-γ (U/ml)
1	AT	0·6	3
2	AT	ND	50
3	AT	<0·3	30
4	AT	<0·3	3
5	CVI	1	100
6	CVI	1	100
7	CVI	<0·3	30
8	CVI	3	158
9	CID	<0·3	<3
10	WA	0·5	31
11	HIgE	<0·3	3

ND, not done.
For other abbreviations, see text.

IFN-γ titration

The amount of IFN released in the supernatants was measured by two independent methods. All supernatants were titrated for IFN activity on human WISH cells by Sindbis virus haemagglutination yield reduction after a single growth cycle (Dianzani, Monahan & Santiano, 1982). A laboratory standard was included in the titrations, and the antiviral activity identified as IFN-γ according to current criteria (Dianzani *et al.*, 1979; 1982). Twenty-eight supernatants from six patients and two controls were also tested by an immunoradiometric assay (IRMA, from Centocor, Malvern, PA) which uses monoclonal antibodies to detect IFN-γ (Chang *et al.*, 1984). All samples were run in duplicate, and the same laboratory standard of the bioassay was used along with the manufacturer's standards. Results were interpolated from the standard curve, and expressed as U/ml.

To explore the presence of soluble inhibitors of IFN-γ antiviral activity in the bioassay, supernatants were mixed with a known IFN-γ preparation and retested to detect any significant interference.

RESULTS

IL-2 and IFN-γ production after PHA stimulation

These were evaluated on 24 h supernatants of PBMC stimulated with PHA and the results obtained are shown in Table 1. Average normal levels of IL-2 after PHA stimulation were $5{\cdot}9 \pm 5{\cdot}1$ U/ml in 20 healthy subjects tested on several occasions. Interleukin 2 production below 0·5 U/ml was considered to be severely reduced. Five out of ten patients studied had undetectable IL-2, and two more only produced low amounts (0·6 and 0·5 U/ml respectively in Patients 1 and 10, Table 1). IFN-γ production induced by PHA was in agreement with that observed after induction by SEB or GO, used for preliminary selection of defective patients. Seven out of 11 cases tested had very low IFN-γ values, compared to normal subjects (all with

Table 2. *In vitro* IFN-γ production (U/ml) by RPMC of normal subjects induced with recombinant IL-2 (rIL-2), galactose oxidase (GO), staphylococcal enterotoxin B (SEB) and their combinations

	Spontaneous	rIL-2	GO	GO+rIL-2	SEB	SEB+rIL-2	A23187
Controls							
1	<3	<3	3	10	30	100	—
2	<3	30	30	100	300	300	—
3	<3	<3	3	3	100	200	—
4	<3	<3	200	1000	500	500	—
5	<3	<3	300	1000	3000	3000	—
6	<3	<3	100	100	100	300	—
7	<3	<3	100	30	500	300	—
8	<3	<3	100	100	1000	300	—
9	<3	10	300	300	1000	100	—
10	10	10	300	500	1000	30	—
11	10	10	100	50	500	500	—
Patients							
1	<3	<3	<3	10	<3	10	5
2	<3	<3	<3	<3	<3	<3	20
3	<10	<10	100	100	300	100	100
4	3	3	100	100	200	300	100
5	<3	10	30	30	100	30	100
6	<3	<3	3	10	100	<3	10
7	<3	<3	100	300	500	1000	300
8	<3	<3	50	100	500	200	30
9	<3	<3	<3	<3	3	30	30
10	<10	<10	<10	<10	<10	<10	<10
11	<3	<3	<3	<3	<3	3	20

Patients numbered as in Table I.

levels above 100 U/ml). When comparing the results of both IL-2 and IFN-γ produced in response to PHA, we observed that patients with defects of cell mediated immunity (AT, CID, WA and HIgE in our series) showed major defects of lymphokine production, and that there was a good correlation between the amount of IL-2 and IFN-γ detectable in culture supernatants ($r=0{\cdot}886$, $P<0{\cdot}01$; Spearman's correlation coefficient $=0{\cdot}769$, $P<0{\cdot}01$, one sided). In fact, Patients 5, 6 and 8, with CVI, produced 1 U/ml of IL-2 or more and they also showed IFN-γ values of 100 U/ml or above (Table 1). Since IL-2 has been shown to enhance IFN-γ release under suboptimal stimulation conditions (Kasahara *et al.*, 1983), we tested whether low production of IFN-γ could be partly restored by addition of IL-2 *in vitro*.

Effect of IL-2 addition on IFN-γ production

We compared the ability to respond to several inducers of IFN-γ with and without the addition of 10 U/ml of rIL-2 in PBMC from 11 normal subjects as opposed to 11 ID patients. The results are shown in Table 2. Spontaneous production of IFN-γ *in vitro* was observed only to a low titre in two controls (Nos 10 and 11, Table 2). None of the other control subjects or patients showed significant IFN-γ production in unstimulated cultures (Table 2). Recombinant interleukin 2 alone proved unable to induce production of IFN-γ in resting unstimulated PBMC from either patients or controls, perhaps with the exception of control subject No. 2 in Table 2. Under the present experimental conditions, SEB was confirmed to be a more potent inducer than GO in all normal controls, with mean levels of 100–3000 U/ml vs 30–300 IU/ml (range). The addition of rIL-2 did not cause any dramatic change of secretion (Table 2). Defective IFN-γ production after GO and SEB stimulation was found in the majority of ID patients (Table 2), namely two with AT (Patients 1 and 2), two with CVI (Patients 5 and 6, both however showing low-normal values of 100 U/ml when induced with SEB) and the remaining three with CID, WA and HIgE (Patients 9 to 11). None of these cases had an absolute absence of T lymphocytes, and their deficiency was therefore a functional one. When we compared these with the results of IL-2 production (Table 1) we could no longer find a relationship between the production of these two lymphokines. As one would predict from this

Table 3. Production *in vitro* of IFN-γ by PBMC from normal controls and primary immunodeficiency patients and their co-cultures

PBMC source	Gamma interferon (U/ml)		
	Spontaneous	GO	SEB
Normal	<10	1000	2000
AT No 3	<10	100	300
WA No 10	<10	<10	<10
Normal+AT	<10	300	3000
Normal+WA	<10	300	300
AT+WA	<10	100	100

Patients are numbered as in Table 1.
For abbreviations see Table 2.

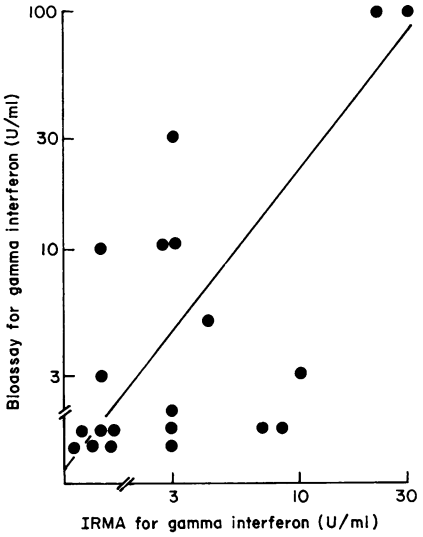

Fig. 1. Comparison of IFN-γ results in supernatants from primary ID patients, measured by both bioassay and IRMA methods. Spearman rank correlation coefficient $r_s = 0{\cdot}521$ ($P < 0{\cdot}05$) linear correlation coefficient $r = 0{\cdot}899$ ($P < 0{\cdot}01$).

observation, the addition of 10 U/ml rIL-2 to GO- and SEB-stimulated PBMC did not induce production of IFN-γ in severely defective ID patients (Table 2). No consistent changes were also found in four patients with near normal IFN-γ levels (Patients 3, 4, 7, 8) since induction is already maximal with both stimuli. Similar findings were obtained in five parents of AT patients (Patients 1, 2 and 4) who showed normal production of IFN-γ and no significant change after addition of rIL-2 (data not shown).

Effect of calcium ionophore on gamma IFN production

Since macrophage- T cell cooperation via soluble factors is an essential step in IFN-γ induction, we wondered if any defect at the level of cellular interaction could be responsible for the defect of IFN-γ production. To investigate this step we stimulated the mononuclear cells with A23187, a bivalent cation ionophore which also induces IFN-γ secretion in the absence of macrophages (Antonelli & Dianzani, 1985). An absolute defect of IFN-γ was confirmed in Patients 1, 2, 6, 10 and 11, with levels below 30 U/ml, and low values were also found in Patients 8 and 9 (Table 2). The other four ID patients produced normal amounts, since IFN-γ levels in seven healthy controls ranged from 30 to 1000 U/ml.

Detection of inhibitors of IFN-γ

The possibility that soluble inhibitors of antiviral action were secreted by 'defective' patients was explored by mixing cell supernatants with a standard IFN-γ preparation. The results obtained in Patients 2, 3, 6 and 11 indicated the absence of soluble inhibitors or suppressive factors of the antiviral activity of IFN-γ (not shown).

Co-cultures of control normal cells with PBMC from two patients in the presence of inducers of IFN-γ were set up in order to investigate the possible role of cell-mediated suppression of IFN-γ production (Table 3). One of the patients (Patient 10) was a non-responder, while the other (Patient 3) produced near normal levels of IFN-γ *in vitro*. PBMC (10^6) of both patients and controls were mixed and stimulated with GO and SEB. The results in Table 3 demonstrate no evidence of cell-mediated suppression, since the different values obtained in comparison with supernatants of each subject alone were within the expected range, taking into account the variation of total number of producing cells. In fact, only a small difference could be observed when cells from a normal producer were compared with the addition of cells from a non-responder.

Comparison of biological and IRMA assays for IFN-γ

Twenty-eight supernatants from six patients and two controls were tested by both the bioassay and the IRMA in order to see whether non-producers were secreting an inactive or altered molecular form of IFN-γ which could still be recognized by monoclonal antibody as immunoreactive. This possibility was discarded after both tests in our hands showed a very close concordance in the measurement of IFN-γ in the supernatants (with $r = 0{\cdot}89$; $P < 0{\cdot}01$) and similar sensitivity. IFN titres obtained in patients are reported in Fig. 1.

DISCUSSION

Two main questions were addressed by our study: (1) whether defective production of IFN-γ and IL-2 in selected primary ID patients were coexistent but independent events, or one defect was a consequence of the other, and (2) whether IFN-γ production was impaired because of (a) defective cellular interactions, (b) active suppression or (c) lack of detectability in our bioassay system.

We have confirmed previous data (Virelizier *et al.*, 1979; Matricardi *et al.*, 1984) on severe deficiency of IFN-γ synthesis using several inducing agents (PHA, SEB and GO), in a subgroup of ID cases, mainly those with defects affecting T cell mediated immunity. Ataxia-telangiectasia (AT) patients showed marked heterogeneity with respect to IFN-production, so this function should not be regarded as a main feature of the disease (Jaspers & Bootsma, 1982; Fiorilli *et al.*, 1983; Paganelli *et al.*, 1984). Common variable immunodeficiency cases displayed a remarkable variability of the results with time of sampling, since all four patients had been previously selected from 20 CVI cases for low IFN production (Matricardi *et al.*, 1984), but one presented borderline and two normal values at the time of this study. Most of these patients also had a defective production of IL-2 *in vitro*, but the expression was not consistently related to the production of IFN-γ induced by different agents. This finding suggested that neither defect was secondary to the absence of the other lymphokine. Direct evidence was obtained by the lack of restoration of IFN-γ production in defective cases by the addition of optimal concentrations of rIL-2. We therefore suggest that the two defects in primary ID are independent and not amenable to correction by administration of IL-2 alone. This is at variance with observations made in patients with AIDS and AIDS-related complex, who are unable to produce both IL-2 and IFN-γ in response to microbial antigens. *In vitro* addition of 10 U/ml of rIL-2 could effectively restore IFN-γ production in some patients with AIDS and in most with AIDS-related complex (Murray *et al.*, 1985). However, using mitogenic stimulation we did not observe this effect, perhaps due to already maximal induction.

The second question was investigated by three sets of experiments. First, we stimulated the cells with calcium ionophore, which induces IFN-γ even in the absence of macrophage/T lymphocyte interaction (Antonelli & Dianzani, 1985) thus bypassing this essential step required for stimulation with all other inducers (PHA, SEB, GO). Since A23187 did not stimulate defective PBMC to secrete IFN-γ, we postulated that other mechanisms were operating in these patients. Experiments carried out with mixtures of normal and defective cells, co-cultured in the presence of inducers, showed that active suppression of IFN-γ production did not account for the lack of IFN production. Indeed these co-culture systems showed that a selective deficiency of T helper cells, which might be replaced by the addition of normal cells, could also be ruled out.

Since IFN-γ shares antiviral properties with other IFNs, but has a predominant immunoregulatory action, we asked whether our detection system, based on the antiviral effect, might be appropriate to identify this lymphokine. Moreover, defective patients might be producing a slightly altered protein, devoid of antiviral activity. We therefore re-assayed all supernatants from deficient cases by a highly specific IRMA based on monoclonal antibodies (Chang *et al.*, 1984), able to measure the presence of immunoreactive IFN-γ irrespective of biological activity. The results showed that no IFN-γ was present in the defective supernatants, in agreement with the lack of antiviral activity *in vitro*.

We could therefore show that defective IFN-γ production in ID patients is a primary defect, independent of the production, cellular interactions and inducing agents of other lymphokines. It is to be stressed that all the cases studied had functional defect, since lack of IFN-γ production was not due to absence of mononuclear cell types capable of production (lymphocytes, NK cells).

Our studies have indicated that calcium transport across the cell membrane, a key event in cell activation (Imboden, Weiss & Stobo, 1985a, b) cannot restore IFN-γ production, so the level of the defect probably lies in inappropriate transcription or translation of IFN-γ gene. This is at variance with what is observed in T-CLL, where stimulation of IFN-γ production could be achieved with A23187, but not with SEB or GO (Pandolfi *et al.*, 1985). Genomic deletion seems to be unlikely, since the gene for IFN-γ is on chromosome 12, which is seldom involved in translocations or deletions even in diseases with high frequency of chromosomal abnormalities, such as ataxia-telangiectasia (Jaspers & Bootsma, 1982; Fiorilli *et al.*, 1985). The independent expression of defective lymphokine release is not entirely surprising, because retrovirus-infected cells may be selectively unable to transcribe IL-2 but not IFN-γ genes into mRNA (Arya & Gallo, 1985) and a dissociation between IL-2 and IFN-γ production has also been described in newborns (Lewis, Larsen & Wilson, 1986).

A preliminary study on families of patients with AT showed that IFN-γ production has no value in discriminating heterozygotes for the genes coding for the phenotypic expression of the disease. This would have been a very useful test if it carried a predictive value, since even defective repair of radiation-induced DNA damage is not able to help for genetic advice. It is to be noted that not all AT patients are defective IFN-γ producers (Paganelli *et al.*, 1984) and all parents, though obligate heterozygotes for AT gene(s), are phenotypically normal responders, irrespective of the status of their affected child. The association of ID with lack of IFN-γ secretion may be due not to genetic abnormalities but to an intrinsic defect of T cell activation and function. This test may help to explain the different severity of expression of ID syndromes, and provide a valid rationale for the therapeutic use of this purified lymphokine.

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