

Original article

Selective insufficiency of IFN- γ secretion in patients with hyper-IgE syndrome

Background: Hyper-immunoglobulin E (IgE) syndrome is a complex immune deficiency characterized by chronic eczematous dermatitis, recurrent staphylococcal infections, pneumatoceles, reduced neutrophil chemotaxis, and variably impaired T cell function. Although decreased interferon- γ (IFN- γ) production in patients with hyper-IgE syndrome is pointed out and known as a cause of reduced neutrophil chemotaxis, precise mechanism of their inadequate production of IFN- γ remains unknown. To elucidate the pathogenesis of the defective production of IFN- γ in patients with hyper-IgE syndrome, we assessed the *in vitro* production and secretion of IFN- γ by peripheral blood mononuclear cells (PBMCs) from patients with hyper-IgE syndrome.

Methods: Chemotaxis of neutrophils, mRNA levels of several cytokines, intracellular production and extracellular secretion of IFN- γ , interleukin-2 (IL-2), and IL-4 by PBMCs from three patients with hyper-IgE syndrome were determined.

Results: The transcription of IFN- γ mRNA and the production of its protein molecules progressed normally. However, selective insufficiency in the secretion of IFN- γ molecules was found in patients with hyper-IgE syndrome. Confocal laser scanning microscopy clearly demonstrated the accumulation of IFN- γ in patients with hyper-IgE syndrome.

Conclusion: We demonstrated that there was a selective insufficiency in the secretion of IFN- γ in patients with hyper-IgE syndrome. We hope that this fact would offer a new paradigm for understanding this disease.

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Hyper-immunoglobulin E (IgE) syndrome is a primary immunodeficiency disease characterized by eczema, recurrent staphylococcal infections, pneumatoceles, reduced neutrophil chemotaxis, and variably impaired T cell function (1–3). Although the cause is not known, elevated serum levels of IgE are uniformly found. It has been hypothesized that elevated serum levels of IgE are associated with a Th1/Th2 imbalance (4–8) and the susceptibility to infections by certain pathogens is thought to be associated with low levels of available interferon- γ (IFN- γ), possibly due to low levels of production. This study was designed to determine the mechanisms of this reduced IFN- γ production in relation to the following four possibilities: (1) there is an insufficiency in the production of IFN- γ , (2) there is a problem within the IFN- γ receptor, (3) the life span of produced IFN- γ is short, (4) there is a problem with post-production IFN- γ secretion.

Material and methods

Clinical characteristics of patients

We studied three patients aged 12 (9), 19 (10), and 20 years (two males and one female), who were diagnosed as having hyper-IgE

syndrome and who had been subject to recurrent staphylococcal pulmonary and skin infections from birth or early childhood. Clinical features of these patients are given in Table 1. The patients uniformly manifested a history of persistent eczema from early childhood, and recurrent cold staphylococcal abscesses, chronic sinopulmonary infections, mucocutaneous candida infections, and a history of bone fractures. One had retained primary teeth and exhibited scoliosis.

All three patients had significantly elevated serum IgE levels ranging from 11 676 to 43 000 IU/ml, and they also had chemotactic defect in the circulating neutrophils. Their lymphocyte number and function were normal. The patients and controls all gave their informed consent to participate in the studies.

Subjects and cell cultures

Peripheral blood mononuclear cells (PBMCs) were obtained from three patients with hyper-IgE syndrome and healthy volunteers by venipuncture. Blood samples were taken during the period when there were no manifest signs of infection. The PBMCs and neutrophils were separated by density gradient centrifugation over LymphoprepTM (Nycomed, Oslo, Norway) or Ficoll-Conray. In most cases, cells were cultured under stimulation with both 10 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma, St Louis, MO) and 500 ng/ml of ionomycin calcium (Nakarai Tesque, Kyoto, Japan) for 4–16 h at 37°C in a 5% CO₂ humidified atmosphere.

Table 1. Clinical characteristics of patients

Patient no.	1	2	3	
Age	19	12	20	
Sex	M	F	M	
Skin abscess	>4	>4	>4	
Pneumonia	>3	>3	>3	
Parenchymal lung anomalies	Pneumatocele	Absent	Bronchiectasis	
Retained primary teeth	>3	None	None	
Scoliosis	22°	<10°	<10°	
Fractures with minor trauma	5	1	3	
Highest eosinophil count (cells/ μ l)	1300	1006	2227	
Characteristic face	Present	Present	Present	
Midline anomaly	Present	Present	Present	
Newborn rash	Present	Present	Present	
Eczema (worst stage)	Severe	Severe	Severe	
Upper respiratory infections/year	>6	>6	>6	
Candidiasis	Fingernails	Fingernails	Fingernails	
Other serious infections	Severe	Severe	Severe	
Severe infection	Present	Present	Present	
Hyperextensibility	Absent	Absent	Absent	
Lymphoma	Absent	Absent	Absent	
High palate	Absent	Absent	Absent	
Highest serum IgE level (IU/ml)	43 000	26 700	11 676	<250
<i>S. aureus</i> IgE (%)	9.1	4.5	8.2	0.2 ~ 0.6
<i>Candida</i> IgE (%)	24	10.5	18	0.3 ~ 0.5
Mite IgE (%)	0.8	0.8	0.7	0.3 ~ 0.6

Chemotaxis of neutrophils

Chemotaxis was studied by a modification of Boyden's procedure (11, 12). A known quantity of polymorphonuclear neutrophils was delivered to a 5 μ m pore size millipore filter (Millipore Corp., Bedford MA) utilizing a cytocentrifuge. The filters were then placed in modified Boyden chambers, and a chemotactic stimulus was added to the attractant side. After incubation, the number of cells migrating completely through the filter was determined by microscope and a chemotactic index was calculated. Recombinant human IFN- γ (Imunomax[®]- γ , Shionogi, Japan) was supplied as an endotoxin-free preparation in lyophilized form. Dose-response experiments revealed excellent activity of this preparation on polymorphonuclear neutrophils in concentrations ranging from 0.0001 to 10 U/ml.

RNase protection assay

The messenger RNA levels of several cytokines in PBMCs were investigated using a ribonuclease protection assay (RPA) system (BD Pharmingen, San Diego, CA). Total cellular RNA was isolated from frozen cell pellets (both stimulated and unstimulated) by RNeasy[®] Mini (Qiagen, Hilden, Germany). The RPA was performed using 5 μ g of RNA samples by RiboQuant[®] human cytokine/chemokine multi-probe template sets kit (hCK-1) (BD Pharmingen). The hCK-1 was constructed from templates of human interleukin-5 (IL-5), IL-4, IL-10, IL-14, IL-15, IL-9, IL-2, IL-13, IFN- γ and the house keeping genes [ribosomal protein L32 (L32)] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). After the probe synthesis was performed by labeling [³²P] UTP, the probe was hybridized with the sample RNA for 16 h, and then digested with ribonuclease at 30°C for 45 min. After stopping the reaction, RNA was collected and poured into the gel mold. Finally, the samples were analyzed by Science Lab 99 Image Gauge with BAS2000 (Fujifilm, Tokyo, Japan).

Flow cytometry studies

Intracellular cytokine production by PBMCs was investigated following cell culture for 4 and 16 h. Mononuclear cell membrane was permeabilized using FACS permeabilizing solution (BD Pharmingen), and incubated with mAbs anticytokine (FITC-conjugated anti-IFN- γ or anti-IL-2 and PE-conjugated anti IL-4 from BD Pharmingen). Cytoplasmic cytokines were analyzed with a FACScan (BD Pharmingen) and CellQuest software (13).

Human cytokine ELIspot assays

Human cytokine ELIspot assay was carried out according to the same procedure described elsewhere (14). Briefly, serial dilutions of a single cell suspension, starting with 10⁵ to 10⁶ cells/well, were incubated on 96 cytokine-coated well plates with PHA (Gibco, Carlsbad, USA) at 37°C for 6 h in a humidified environment with 5% CO₂. After washing, cells were incubated with biotinylated anticytokine antibody for 2 h and then with avidin-conjugated alkaline phosphatase (Vector, Burlingame, CA, USA) for 1 h at room temperature.

The cytokine secreted by single cells was visualized by the addition of a solution of BCIP/NBT (KPL, Gaithersburg, MD, USA). The colorimetric reaction was halted after 20 min, and spots were counted under 40 $\times$.

Confocal laser scanning microscopy (CLSM) and electron microscopy (EM)

The PBMCs (1 $\times$ 10⁶), before and/or after cell culture, were employed by cytopspin technique, placed on glass slide, and then fixed in 4% paraformaldehyde (PFA). These cells were incubated with monoclonal antibodies (monoclonal anti-human IFN- γ anti-

body, R & D, NJ) or normal rabbit serum overnight. The cells were subsequently incubated overnight with a horseradish peroxidase conjugated rabbit-anti-mouse immunoglobulin antibody solution (Amersham Pharmacia Biotech, Buckinghamshire, UK) supplemented with 1% BSA-PBS. 3,3'-diaminobenzidine(DAB), together with H_2O_2 , was used for the demonstration of peroxidase reactivity. The cells were post-fixed in 2% osmium tetroxide for 1.5 h, dehydrated in a series of graded ethanol solutions, and embedded in resin Quethol 812 by placing the capsule upside down on the positively staining cells. After polymerization, ultra-thin sections were examined by EM. Intracellular immunohistochemical localizations of IFN- γ were demonstrated by CLSM reflectance mode with a helium-neon laser (wavelength of 543 nm) (red color). The cell structures were visualized by CLSM transmittance mode with an argon-ion laser(wavelength of 488 nm) (blue color) and nucleus were detected by CLSM fluorescence mode with a helium-neon laser(wavelength of 543 nm) (green color, nucleus-methyl green). The imaging was achieved using CLSM (LSM-410, Carl Zeiss, Jena, Germany) (15).

Results

Neutrophil chemotaxis

It has been reported that children with hyper-IgE syndrome have a profound defect in neutrophil granulocyte chemotaxis (5, 11). Thus, the recurrent staphylococcal abscesses in these patients might be related to defective neutrophil chemotaxis. Therefore, we investigated neutrophil chemotaxis in patients with hyper-IgE syndrome and determined the effect of recombinant human IFN- γ *in vitro*.

All three patients had markedly decreased neutrophil random migration. Phagocytosis and bactericidal activity were normal (data not shown). After incubation with IFN- γ , improvement in chemotactic responsiveness was observed in the neutrophils of each of the patients. Also the chemotactic response of the patients' cells increased in a dose-dependent manner. In contrast, the neutrophils from the control subjects showed no significant increase in chemotaxis after incubation with IFN- γ (Fig. 1).

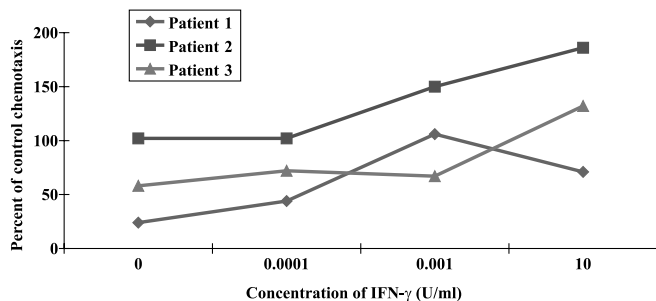


Figure 1. Effect of human IFN- γ on chemotactic responses of neutrophils from three patients with hyper-IgE syndrome. After incubation with IFN- γ (0–10 U/ml), improvement in chemotactic responsiveness (in a dose-dependent manner) was observed in the neutrophils of each of three patients. Neutrophils from the control subjects showed no significant increase in chemotaxis after incubation with IFN- γ .

These data suggest that the decreased neutrophil chemotactic responses were not due to the abnormal neutrophil function but to the insufficient production of IFN- γ in patients with hyper-IgE syndrome.

IFN- γ receptor

Recently, it was shown that nonfunctional or dysfunctional IFN- γ receptors were present in patients with an absent or diminished response to IFN- γ (16, 17). These patients are known to be susceptible to infection, and are also thought of as a new phenotype of primary immunodeficiency (18). To exclude IFN- γ receptor deficiency in patients with hyper-IgE syndrome and to determine whether IFN- γ signaling was intact, IFN- γ receptor expression on PBMCs in patients with hyper-IgE syndrome was characterized. Immunofluorescence studies showed that the receptor was present and there was no difference in the intensity of expression of the IFN- γ receptor between the patients and the controls (data not shown).

Messenger RNA analysis

From the study described above, there seemed to be a deficiency of IFN- γ in patients with hyper-IgE syndrome. We have previously reported (6) that there is a Th1/Th2 imbalance in patients with hyper-IgE syndrome and the defective production of IFN- γ might be related to the pathogenesis of this disease. Also, Del Prete et al. (19) and Paganelli et al. (20) have reported that IFN- γ production *in vitro* was severely reduced in the patients with hyper-IgE syndrome, and this IFN- γ deficiency may be responsible for some of the features displayed by patients with hyper-IgE syndrome, including IgE levels and susceptibility to infections. Based on this evidence, we conjectured that some deficiency lies within the process of production, intracellular transportation, and secretion of IFN- γ . Therefore, we first examined the production of IFN- γ , especially the gene expression of IFN- γ . The mRNA was extracted from PBMCs with or without stimulation with PMA and subjected to RPA analysis. The mRNA levels of IFN- γ were comparative in patients with hyper-IgE syndrome to those of controls. No differences were found in the gene expression of IL-2 between the patients with hyper-IgE syndrome and the controls (Fig. 2).

Immunofluorescence and flow cytometry

The gene expression of IFN- γ was intact in patients with hyper-IgE syndrome. We next investigated intracellular production of IFN- γ by cytoplasmic staining in CD4⁺ T cells. No significant difference was detected in the percentages of CD4⁺ T cells expressing intracellular IFN- γ protein between the patients with hyper-IgE syndrome and the controls after 4 h of incubation. Also the levels of intracellular production of IL-4 and IL-2 were the same in patients with hyper-IgE syndrome and

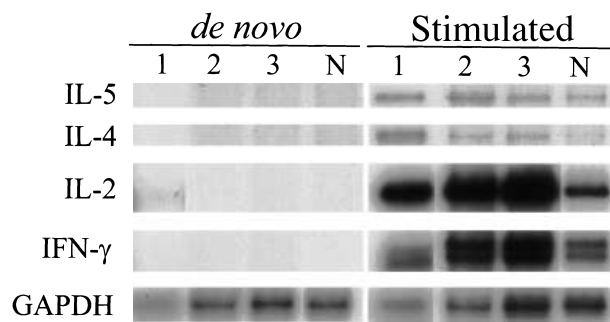


Figure 2. Autoradiogram of RPA showing mRNA expression of cytokines from PBMCs of three patients with hyper-IgE syndrome and controls. No differences were found in the gene expression of IFN- γ , IL-2, and IL-4 between three patients with hyper-IgE syndrome and controls. 1,2,3; patients N; control de novo; without stimulation with PMA stimulated; after stimulation with PMA.

in controls. These data demonstrate that intracellular production of IFN- γ was normal in patients with hyper-IgE syndrome. After 16 h of incubation, the quantity of intracellular IFN- γ significantly decreased in the controls, probably due to excretion to the medium. However, intracellular IFN- γ was persistently detected in PBMCs from patients with hyper-IgE syndrome. These data suggest that there might be inadequate secretion of IFN- γ from PBMCs to the medium in patients with hyper-IgE syndrome. In comparison, intracellular IL-4 was almost depleted both in the controls and in patients with hyper-IgE syndrome after 16 h of incubation.

Frequency of cytokine secreting cells by ELISpot assay

The intracellular production of IFN- γ in cytoplasm turned out to be normal in patients with hyper-IgE syndrome. Therefore, we utilized ELISpot assay to evaluate the cytokines released from PBMCs with or without stimulation with phytohemagglutinin (PHA) in patients with hyper-IgE syndrome and in controls. Significant decreases were detected in the number of PBMCs which spontaneously secreted IFN- γ in patients with hyper-IgE syndrome compared with PBMCs in controls. Similarly, significantly fewer PBMCs from patients with hyper-IgE syndrome were induced by PHA to secrete IFN- γ , compared with controls. In the controls, 328/10⁶ cells apparently secreted IFN- γ when stimulated with PHA. In contrast, only 0 to 63/10⁶ cells were observed to secrete IFN- γ in the patients with hyper-IgE syndrome. As for IL-4 and IL-2, no significant patient/control differences were found in the number of secreting cells (Table 2).

Confocal laser scanning microscopy and electron microscopy

Our study demonstrated that the transcription of IFN- γ mRNA and the production of its protein molecules progressed in a normal fashion in PBMCs of the patients

Table 2. Cytokine secreting cells by ELISpot assay

Patient	PHA(-)			PHA(+)		
	IFN- γ	IL-4	IL-2	IFN- γ	IL-4	IL-2
1	5	0	1	0	30	18
2	9	0	3	63	27	16
3	0	0	4	0	30	21
Control	100	0	10	328	27	22

Freshly isolated PBMCs were analyzed for cytokine production after incubation with PHA for 16 h or without PHA.

Number of cells producing each cytokine per 10⁶ PBMC.

with hyper-IgE syndrome. However, both results from flow cytometry analysis and ELISpot assay indicated that there must be selective insufficiency in the secretion of IFN- γ in patients with hyper-IgE syndrome. To confirm this phenomenon in patients with hyper-IgE syndrome (and to visualize selective insufficiency in the secretion of IFN- γ in PBMCs), we used CLSM.

First, we investigated intracellular expression of IFN- γ molecules without any stimulation. To produce IFN- γ under normal circumstances, PBMCs need to be activated by mitogens. This means that in the resting phase of PBMCs, IFN- γ molecules cannot be detected. However, as shown in Fig. 3A, IFN- γ molecules were detected in patients with hyper-IgE syndrome even in the resting phase of PBMCs. No molecules of IFN- γ were evident in the cells of normal controls (Fig. 3C). After stimulation with PMA, significant differences were visualized in the amount of IFN- γ as positive staining inside the PBMCs between patients with hyper-IgE syndrome and the controls (Fig. 3B and D). Moreover, IFN- γ molecules were distributed in the perinuclear areas through the cellular organelles to the plasma membrane area (Fig. 4).

Taken together, these findings suggest that selective insufficiency in the secretion of IFN- γ may be the central issue in the immune deficiency associated with hyper-IgE syndrome.

Discussion

Our study indicated that the transcription and translation of IFN- γ in the PBMCs of patients with hyper-IgE syndrome progressed in a normal manner. However, selective insufficiency in the secretion of this cytokine was observed in these patients and seemed likely to be a source of major problems. Confocal laser scanning microscopy clearly demonstrated the accumulation of IFN- γ in the cytoplasm of lymphocytes in patients with hyper-IgE syndrome.

Hyper-IgE syndrome is an immunodeficiency disease characterized by chronic eczematous dermatitis, recurrent pyogenic infections of the skin, characteristically caused by staphylococcus aureus, and mucocutaneous candidiasis (1–3). The most common manifestations of immune

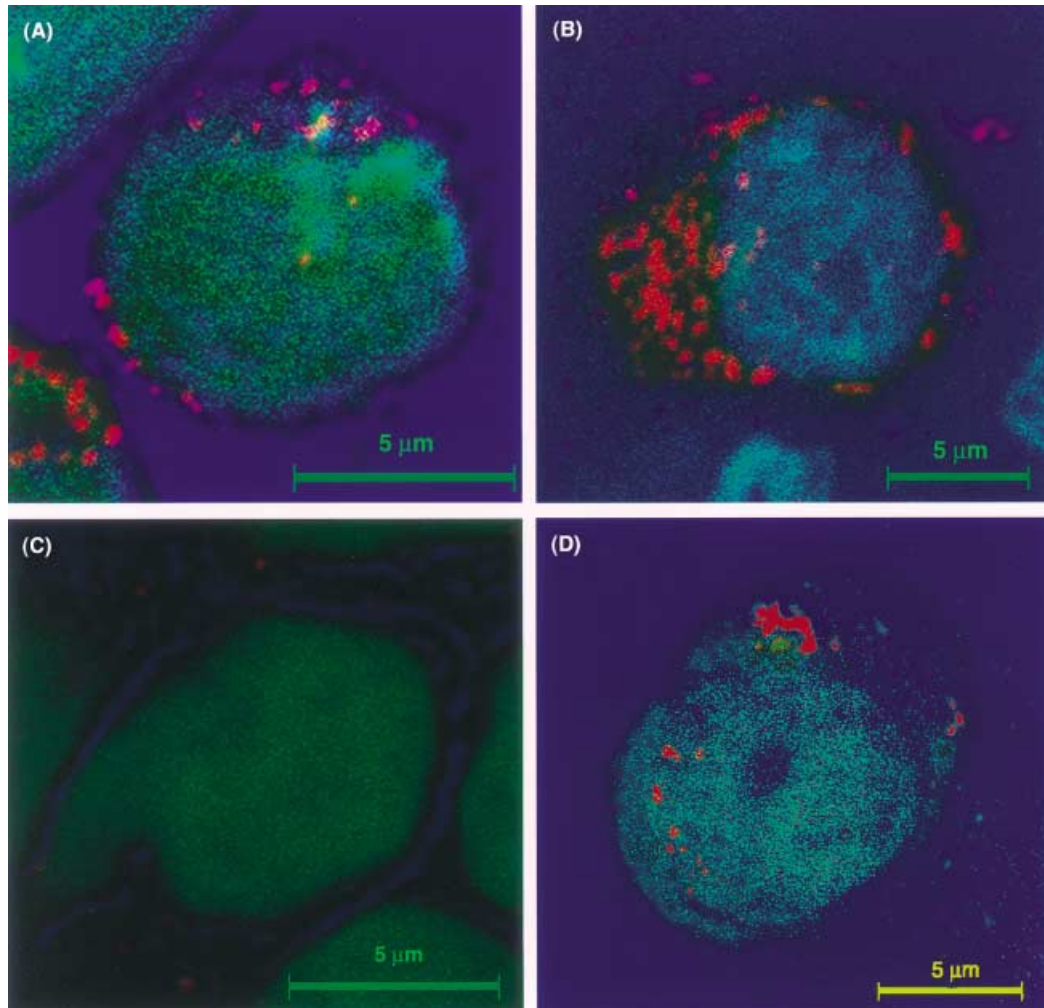


Figure 3. By confocal laser microscopy, significant accumulation of IFN- γ was observed in the PBMCs of patients with hyper-IgE syndrome after stimulation with PMA. IFN- γ ; (red) nucleus; (green) (A) PBMC of patient without stimulation with PMA, (B) PBMC of patient with stimulation with PMA, (C) PBMC of control without stimulation with PMA, and (D) PBMC of control with stimulation with PMA.

dysfunction in patients with hyper-IgE syndrome are markedly elevated serum IgE, defective granulocyte chemotaxis, and decreased production of IFN- γ (2). A causal relationship among clinical features, immune dysfunction, and excessive production of IgE in hyper-IgE syndrome has not yet been established.

Recent advances have provided a great deal of information about the pathogenesis of hyper-IgE syndrome. The increase in IgE production is probably sustained by complex mechanisms regulating T and B cell interactions, which involves many local factors. The imbalance in cytokine production, and mainly the low IFN- γ secretion, is thought to be responsible for the shift to Th2 cell dominance noted in these diseases and may account for the susceptibility to infections and markedly elevated serum levels of IgE (4, 5). However, not all studies were consistent in identifying a Th1/Th2 imbalance (4–8).

Del Prete et al. (19) have reported that T cells from patients with hyper-IgE syndrome were found to produce significantly lower concentrations of IFN- γ in response to stimulation with PHA than did T cells from age-matched healthy controls. As IFN- γ exerts an inhibitory effect on the synthesis of IgE and plays an important role in inflammatory reactions, the defective production of IFN- γ may be responsible for hyperproduction of IgE, and the defect of IFN- γ may contribute to the undue susceptibility to infections seen in patients with hyper-IgE syndrome.

Hill et al. (5, 11, 21) noted that patients with hyper-IgE syndrome had a profound defect in neutrophil chemotaxis. The defect of IFN- γ has presented a mechanism for impaired chemotaxis of neutrophils, which was responsible for the undue susceptibility to bacterial and fungal infections seen in patients with hyper-IgE

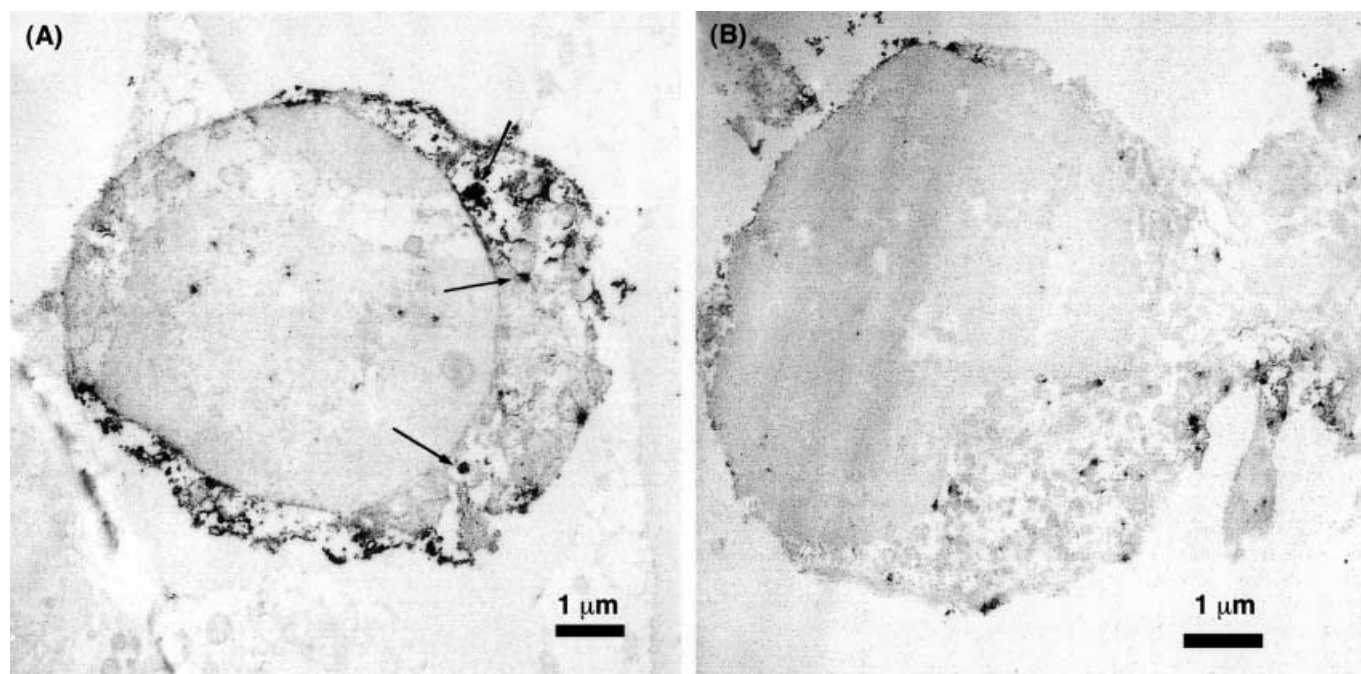


Figure 4. Immunohistochemical localization of IFN- γ in PBMC by immunoelectron microscopy. Significant accumulation of IFN- γ (arrows) was observed in the PBMCs of patients with hyper-IgE syndrome. (A) Patient with hyper-IgE syndrome, (B) control.

syndrome. Incubation of cells from patients with hyper-IgE syndrome with IFN- γ improved the chemotaxis of neutrophils (2, 5, 11). In addition, administration of recombinant IFN- γ is known to improve clinical course of such patients (12, 22).

Furthermore, therapy with recombinant human IFN- γ improves the clinical course of patients with hyper-IgE syndrome (9, 22). So defective *in vitro* production of IFN- γ and the defect in neutrophil chemotactic responsiveness might explain the pathogenesis of at least the susceptibility to infection associated with this syndrome.

Children with hyper-IgE syndrome are prone to long-bone fractures. The bone density of these patients has been reported to be reduced compared with age-matched controls. All of our patients with this syndrome had a history of fractures, and two of three patients had a history of recurrent fractures. Bone fragility in hyper-IgE syndrome is associated with an imbalance in cytokine secreting lymphocyte subpopulation (23, 24). Cytokines are known to be involved in the regulation of osteoclastogenesis and therefore in bone resorption. In patients with hyper-IgE syndrome, the serum levels of PGE₂ were higher and the serum levels of IFN- γ were abnormally low (23, 24). As IFN- γ inhibits bone resorption, and bone degradation is also associated with the spontaneous release of high levels of PGE₂ from activated monocytes, increased bone resorption caused by deficient secretion of IFN- γ might be responsible for the cortical bone loss that leads to a higher incidence of fractures.

Studies of cytokine production by mitogen-stimulated peripheral blood cells of patients with hyper-IgE

syndrome resulted in several distinct findings. Recently, Chehimi et al. (8) pointed out that impaired inflammatory response of this syndrome is due to decreased expression of certain chemokines and cytokines; however, Borges et al. (4) have suggested that decreased IFN- γ production is due to an impaired response to IL-12. In this study, to determine the mechanisms of reduced IFN- γ production, we examined the transcription of mRNA, and the translation and secretion of IFN- γ molecules in lymphocytes. Transcription of mRNA, and the translation of IFN- γ progressed normally. However, a selective deficiency in extracellular secretion of IFN- γ molecules was demonstrated by CLSM, and electron microscopy. In patients with hyper-IgE syndrome, IFN- γ molecules were detected even in the resting phase of PBMCs. After 4–16 h of stimulation with PMA, IFN- γ molecules were still retained in cells. However, no apparent molecules of IFN- γ was detected in the controls. Electron microscopy also demonstrated the accumulation of IFN- γ molecules in PBMCs of patients with hyper-IgE syndrome.

We suggest two possible mechanisms to explain selective insufficiency in IFN- γ secretion. First, some part of the secretory pathway of IFN- γ may be selectively impaired though the mechanisms of intracellular transportation. These secretory mechanisms and secretion of IFN- γ are still to be elucidated. Secondly, the structure of IFN- γ itself may be faulty.

Recently, intracellular protein targeting is emerging as an important approach to elucidating the various mechanisms of the immune system. For example, mechanisms of secretion of immune active molecules,

intracellular protein transportation in antigen presentation, receptor presentation and internalization are under study. Some congenital diseases such as Chediak-Higashi syndrome, Griscelli syndrome, and Hermansky-Pudlak syndrome arise from defects in proteins affecting secretory lysosome release (25). Similarly, there might be an impairment in intracellular transport and secretion of IFN- γ in patients with hyper-IgE syndrome. Consequently, intracellular protein targeting and investigation of the secretion mechanisms seem to be the most promising avenues for elucidating the pathogenesis of this disease.

Finally, we demonstrated that there was a selective deficiency in the secretion of IFN- γ in patients with

hyper-IgE syndrome. We conclude that this fact may contribute to the deficiency of cell-mediated and nonspecific immunity observed in patients suffering from this syndrome and may also be of key importance in the pathogenesis of immune abnormalities of hyper-IgE syndrome.

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