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Defective host defence mechanisms in a family with hypocalciuric hypercalcaemia and coexisting interstitial lung disease

J. AUWERX, M. BOOGAERTS, J. L. CEUPPENS & M. DEMEDTS *Department of Medicine, University Hospitals, Katholieke Universiteit Leuven, Belgium*

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SUMMARY

An extensive *in vitro* investigation of the host defence system was performed in 11 sibs of a large kindred with unexplained combination of familial hypocalciuric hypercalcaemia (FHH), interstitial lung disease and increased susceptibility to respiratory infections. The impairment of host defence mechanism was most likely related to granulocyte dysfunction. A severe myeloperoxidase deficiency was the most consistent granulocyte defect ($P < 0{\cdot}001$) and it was associated with a relatively diminished chemiluminescence ($P < 0{\cdot}001$). Moreover, a significantly diminished antistaphylococcal phagocytic ($P < 0{\cdot}001$) and killing ($P < 0{\cdot}001$) activity was found which in the absence of any opsonizing defect implicates an intrinsic granulocyte dysfunction. We found no abnormalities in number of B and T lymphocytes nor in the balance between helper and suppressor cells determined with monoclonal antibodies. Despite the recurrent infections no elevations of the immunoglobulin subclasses were found. The relationship between the inherited FHH, interstitial lung disease and susceptibility to respiratory infections remains obscure. It is, however, clear that impairment of the host defence might contribute to a decreased life expectancy in this family.

Keywords hypercalcaemia granulocyte function interstitial lung disease parathyroid hormone familial disease

INTRODUCTION

Hypocalciuric hypercalcaemia (FHH) is a familial condition characterized by elevated serum calcium levels without concomitant increase in urinary calcium excretion or abnormal concentrations of calcium regulating hormones (Foley *et al.*, 1972; Marx *et al.*, 1978). It is inherited according to an autosomal dominant pattern (Foley *et al.*, 1972; Marx *et al.*, 1978). Though most authors report a decreased renal and parathyroid reponsiveness to extracellular calcium, the exact cause of this apparently benign disease remains unknown.

We have reported on a large kindred with an unexplained combination of FHH and interstitial lung disease (Demedts *et al.*, 1979; Auwerx *et al.*, 1985). The interstitial lung disease was histologically proven by open lung biopsy in three subjects (including cases 1 and 2 of the actual study), two of whom (including case 1) recently died of an acute exacerbation of the respiratory insufficiency and an overwhelming pulmonary infection. The interstitial lung disease was demonstrated in many other family members by lung function studies. Histopathological, biochemical, enzymatological and immunological examinations excluded sarcoidosis or other

Correspondence: M. Demedts, Universitair Ziekenhuis, Weligerveld 1, B-3041 Pellenberg, Belgium.

known diseases as cause of the abnormalities. Up to now 43 of the family members have been examined (M. Demedts *et al.*, 1985).

In about 65% of the family members an increased frequency of respiratory infections was documented. In 11 of them a thorough study of *in vitro* host defence mechanisms, i.e. granulocyte function, lymphocyte function, complement factors and other serum proteins, was carried out.

MATERIALS AND METHODS

Patients. Eleven family members (six males, five females) were investigated in the winter of 1982–1983 after informed consent was obtained. The ages, pulmonary function and data on calcium metabolism are shown in Table 1. All were regular cigarette smokers except cases 1 and 10. Eight subjects (Nos 1, 2, 4, 5, 6, 8, 10, 11) suffered from more than three respiratory infections per year and two subjects (Nos 7, 9) from more than two, while only one subject (No. 3) did not clearly present such an increased liability. These infections consisted mainly of rhinitis, sinusitis, bronchitis and in some of otitis media. At the time of the present investigation none of the patients was clearly infected.

Granulocyte functional profile. Blood polymorphonuclear neutrophils (PMN) with more than 98% purity (2% mononuclear) and less than 5% platelet contamination were isolated from heparinized (5 u/ml) blood as previously described (Boogaerts *et al.*, 1983). Myeloperoxidase content of blood neutrophils was assayed using a horse radish peroxidase standard (Fehr & Jacob, 1977). Enzyme activity was expressed in units MPO/2×10^6 PMN. Superoxide generation was quantitated spectrophotometrically at 550 nm by the superoxide dismutase sensitive reduction of ferricytochrome C. Zymosan activated by autologous serum (SAZ, 0·5 mg/10^7 PMN) was used as standard stimulant. Results of duplicate samples were averaged and expressed as nanomoles cytochrome C reduced per 2×10^6 PMN in 30 min. Light emission (chemiluminescence) in phagocytosing (zymosan, opsonized by autologous serum) granulocytes was monitored by measuring scintillation counts in the out-of-coincidence mode (Nelson *et al.*, 1977). Results are expressed as stimulation index (maximal stimulation/background counts) at time of peak stimulation. PMN chemotaxis was evaluated by migration under agarose technique (Nelson, Quie & Simmons, 1975). Autologous ZAS (zymosan activated serum, 30′ at 37°C) and FMLP (formyl-methionyl-leucyl-phenylalamine) were used as stimulants. Results were expressed as chemotactic index (maximal chemotaxis/random migration) or as actual distance in μm, migrated by granulocytes under maximal complement (ZAS) or FMLP-stimulation after 3 h. Phagocytosis and

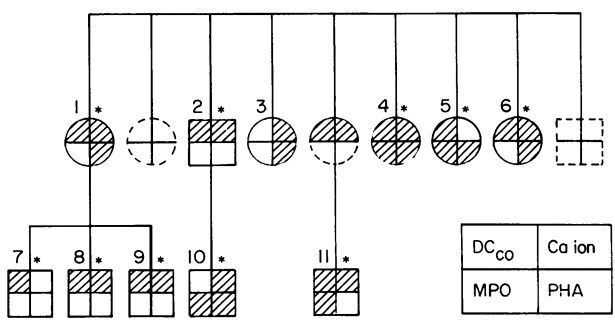

Fig. 1. Pedigree of a limited part of the family. Males indicated by squares, females by circles. Shaded areas represent pathology. Non-investigated subjects or non-investigated aspects indicated by broken lines. Upper left: diffusing capacity for CO < 75% predicted; upper right: hypercalcaemia, ionized calcium > 1·25 mmol/l or total calcium > 2·63 mmol/l (when no ionized calcium was available); lower left: myeloperoxidase content < 10·21 u/2×10^6 PMN; lower right: phagocytosis < 84·5% *Staphylococcus aureus* in 20 min. * = Frequent respiratory infections.

Table 1. Patient characteristics, pulmonary function and studies concerning calcium metabolism

No.	Age (years)	Sex	Total calcium (mmol/l)	Ionised calcium (mmol/l)	Calcium excretion (mmol/24h)	PTH (pg/ml)	25 OH-Vitamin D_3 (ng/ml)	FEV_1% VC (%)	TLC (% pred)	DL_{CO} (% pred)	DL_{CO}/VL (% pred)
1	58	F	2·58	1·53	1·40	105	14	84	56	43	79
2	52	M	2·78	1·39	2·90	65	25	44	112	87	79
3	50	F	2·93	—	1·35	80	25	77	105	81	90
4	43	F	2·18	1·27	2·10	50	22	87	75	63	86
5	42	F	2·30	—	4·37	50	14	81	84	55	70
6	39	F	2·90	1·27	—	50	15	82	77	67	89
7	33	M	2·33	1·21	2·48	55	35	86	88	64	82
8	32	M	2·68	1·44	1·30	70	27	78	95	65	76
9	29	M	2·85	1·40	0·94	65	31	85	88	71	87
10	27	M	2·73	1·39	3·30	70	42	84	90	79	100
11	21	M	2·58	1·34	2·50	60	27	85	103	65	67
Mean	39	—	2·63	1·36	2·30	65	25	79·5	88·5	67·3	82·4
s.d.	11	—	0·25	0·10	1·15	16	9	12·1	15·7	12·2	9·5
Normal ranges			2·25–2·63	1·00–1·25	2·5–10·0	< 100	7–60	> 70	> 80	> 75	> 75

PTH = parathyroid hormone; FEV_1 = forced expired volume in the first second; VC = vital capacity; TLC = total lung capacity; DL_{CO} = diffusing capacity (measured by single breath CO technique).

Control population is described in Materials and methods section and in Auwerx *et al.* (1985).

intracellular killing capacity of granulocytes were assayed using a pour plating technique (Tan, Watanakuwakona & Phair, 1971). Results are expressed as percentage of *Staphylococcus aureus* phagocytosed and percentage *Staph. aureus* killed after 20 min. The killing/phagocytosis index is an accurate measure of granulocyte antibacterial efficiency.

The values were compared with those, obtained in the same period, in 108 healthy control subjects with ages ranging between 15 and 65 years.

Immunological tests. IgA, IgG, IgM and complement factor C_3 were determined with a Hyland laser nephelometer according to the manufacturers instructions. IgE was determined with the PRIST method (Pharmacia). C_1-esterase inhibitor, α-1 antitrypsin and α-2 macroglobulin were estimated with radial immunodiffusion (Behringwerke). C_{3d} was determined on EDTA containing plasma (Perrin, Lambert & Miesscher, 1975). CH_{50} was evaluated with a haemolytic assay according to Vargues (1967). Mononuclear cells were isolated from heparinized blood on Ficoll-hypaque density gradients. For staining of the lymphocytes with monoclonal antibodies a two-step procedure was used. Fluorescein-labelled goat anti-mouse-IgG and Rhodamine-labelled goat anti-mouse-IgM (Nordic, Breda, Netherlands) were used as second label antibodies. The percentage fluorescent cells was determined on at least 200 cells with a Leitz Dialux microscope, using phase contrast to evaluate lymphocyte morphology. B-cells were counted by means of immunofluorescence with fluorescein-labelled anti-IgM. Rosette formation with sheep erythrocytes (after incubation at 4°C overnight) was used to determine the percentage T cells. Monoclonal antibodies were used for subtyping T cells: anti-Leu-2a specific for cytotoxic suppressor T cells (T_{CS}) and anti-Leu-3a for helper inducer cells (T_{HI}) (Ledbetter *et al.*, 1981), and anti-HNK which reacts with natural-killer and killer cells (Abo & Balch, 1981). The values of the lymphocyte subpopulations were compared with those of 31 healthy controls (age $\pm$ 1 sd: 31 $\pm$ 8 years); for immunoglobulin and complement levels the normal values given by the companies providing the test kits were used.

Other tests. A description of the measurements of total calcium, calcium excretion, parathyroid-hormone and 25-hydroxyvitamin D_3 is given by Auwerx *et al.*, (1985). Yet, normal ranges of PTH and ^{25}OH-D_3 are changed, due to an adaptation of the method. Ionized calcium was determined by a potentiometric method using an ionselective electrode. Normal values for total calcium, ionized calcium, calcium excretion, parathyroid hormone and ^{25}OH-D_3 were obtained in 81 healthy adult persons (mean age 37 years, range 18–64 years).

Lung function tests included the measurement of vital capacity (VC), forced expired volume in 1 sec (FEV_1), total lung capacity (TLC) and single breath diffusing capacity for CO (DL_{CO}). Methods and control groups are outlined by Auwerx *et al.* (1985).

Statistics. All results are expressed as the mean $\pm$ s.d. unless indicated otherwise. To assess statistical significance Student's *t*-test and correlation coefficients were used.

RESULTS

Granulocyte functional profile

The results of granulocyte functional tests, carried out in ten patients, are represented in Table 2. Statistically significant differences when compared with controls were found for myeloperoxidase content, chemiluminescence, phagocytosis and killing of *Staph. aureus*. No direct correlation was found between the frequency of infections and severity of granulocyte dysfunction. Yet, the three patients with only slight or no increased liability for respiratory infections (cases 3, 7, 9) tended to perform slightly better (especially for killing of *Staph. aureus*) than the others.

Immunological profile

Results of studies of cellular and humoral immunity are represented in Table 3.

Total number of T cells (i.e. E Rosette$^+$) was normal as well as the distribution of T lymphocyte subtypes, determined with monoclonal antibodies. Total haemolytic complement activity was significantly elevated ($P < 0{\cdot}001$) as well as C_1-esterase inhibitor ($P < 0{\cdot}05$). When compared to matched control subjects our patients had decreased concentrations of IgA and IgM.

Table 2. Granulocyte functional profile

Patient	Myeloperoxidase content (u/2 × 10^6 PMN)	Superoixide generation (mmol/2 × 10^6 PMN/30 sec.)	Chemilumi-nescence (index)	Phagocytosis (%-*Staph. aureus*/20 sec.)	Killing (%-*Staph. aureus*/20 sec.)	Killing/ Phagocytosis index	Chemotactic index (ZAS)	Chemotactic index (FMLP)
1	9·91	17·60	9·80	79	62	0·78	5	4·2
2	11·63	23·88	9·42	78	68	0·87	3·8	4
3	9·71	18·19	8·85	75	71	0·94	—	—
4	6·90	19·90	7·33	62	55	0·88	—	—
5	6·39	17·06	5·50	72	59	0·81	—	—
6	9·25	22·74	10·69	71	49	0·69	5	5·6
7	9·60	20·47	11·47	86	81	0·94	4	3·7
8	10·09	15·92	9·34	89	69	0·77	3·2	3·7
9	11·63	23·88	9·42	78	68	0·87	3·8	4
10	7·59	12·51	8·69	74	62	0·82	5·2	5·2
11	8·28	19·9	11·16	79	70	0·88	3·3	4·6
Mean ± s.d.	8·94 ± 1·62	18·76 ± 3·35	9·23 ± 1·8	76·5 ± 7·7	64·6 ± 9·1	0·84 ± 0·08	4·2 ± 0·8	4·4 ± 0·8
Normal values Mean ± s.d.	12·15 ± 1·94	21·43 ± 4·43	12·13 ± 3·94	88·9 ± 4·4	79·9 ± 5·2	0·90 ± 0·04	3·8 ± 1·02	3·8 ± 1·2
P value	< 0·001	NS	< 0·001	< 0·001	< 0·001	< 0·05	NS	NS

Normal values were obtained in the same period from 108 healthy subjects (ages 15–65 years).

Table 3. Cellular and humoral immunity

	Patients (mean ± 1 s.d.)	Normal (mean ± 1 s.d.)	Differences
E Rosette + (T lymphocytes)%	74 ± 7	71 ± 7	NS
Leu-3a (helper cells) %	49 ± 12	51 ± 9	NS
Leu-2a (suppressor cells) %	25 ± 5	25 ± 7	NS
Leu-7a (natural killer cells) %	13 ± 6	12 ± 7	NS
B lymphocytes	14 ± 11	8 ± 3	NS
CH_{50} u/ml	567 ± 97	432 ± 74	$p < 0{\cdot}001$
C_1 esterase inhibitor mg/100 ml	35 ± 8	19 ± 9	$p < 0{\cdot}001$
C_3 mg/100 ml	126 ± 13	116 ± 20	$p < 0{\cdot}05$
C_{3d}	3·6 ± 1·4	< 3·2	NS*
IgA mg/100 ml	194 ± 130	214 ± 102	NS
IgG mg/100 ml	908 ± 197	1107 ± 268	$p < 0{\cdot}005$
IgM mg/100 ml	100 ± 61	165 ± 88	$p < 0{\cdot}005$
IgE E/ml	61 ± 47	< 120	NS
α-1 antitrypsine mg/100 ml	277 ± 52	289 ± 62	NS
α-2 macroglobuline mg/100 ml	226 ± 92	273 ± 115	NS

*C_{3d} was increased in three out of six patients in whom it was determined.
Control population is outlined in Materials and Methods section.

DISCUSSION

Our investigation of 11 patients of a large family with FHH and interstitial lung disease, clearly demonstrates an impairment of host defence in these subjects.

Granulocyte dysfunction was present in almost all investigated patients. The most consistent defect proved to be a relative myeloperoxidase deficiency. Since chemiluminescence is not only dependent on generation of reactive oxygen species (e.g. superoxide), which was low normal, but also on myeloperoxidase content, which was decreased, the latter may be the main cause of the diminished chemiluminescence response. Moreover, a significantly decreased phagocytotic ability was found in the absence of any opsonizing defect, implicating an intrinsic granulocyte dysfunction. However, the chemotactic response towards autologous activated complement components (ZAS, zymosan activated serum containing mainly C_{3a} and C_{5a}) as well as FMLP (bacterial wall analogue) was normal. This is strong evidence against recurrent infections being responsible for the abnormalities in granulocyte function. Absence of infection at the time of study also excludes 'deactivation' of granulocytes due to cellular receptor blockade by endogenous inflammatory products. Immediate drug effects on neutrophil function are, furthermore, unlikely since most of the subjects did not use medication at the time of the present investigation. As yet, no single cellular defect can be put forward as the primary cause of neutrophil dysfunction in this family.

Both normal and abnormal findings are seen in membrane-receptor mediated functions: stimulation with complement factors results in a normal chemotactic (e.g. C^{5a}) response, but an impaired phagocytotic response. The granulocyte dysfunction in this family may thus be linked partially to a relative myeloperoxidase deficiency, partially to an intrinsic motility defect resulting in defective phagocytosis.

Phagocytosis first requires the specific binding of a ligand to the receptor on the granulocyte surface. Sudden mobilization of calcium and activation of the actin-myosin microfilaments will follow receptor activation. This process requires large amounts of ATP generated by glycolysis. Finally, the granulocyte will engulf the particle in a vacuole through a zipper-like process.

Although, calcium seems to play an important regulating role in phagocytosis, we cannot conclude that the abnormal calcium metabolism seen in FHH is responsible for the defective phagocytosis. Indeed, in our *in vitro* studies, granulocytes are repeatedly washed in normal media, which excludes the presence of excessive amounts of extracellular calcium as the cause of the granulocyte dysfunction. The normal chemotactic response—which also requires a normal intracellular calcium metabolism—pleads further against a role of this cation in the granulocyte dysfunction. Interference with ATP metabolism is also able to alter phagocytosis. Consequently phagocytosis is blocked by the raising of intracellular cAMP levels. Higher urine and plasma levels of cAMP are reported in FHH, after parathyroid hormone infusion (Marx *et al.*, 1980; Heath & Purnell, 1980). Basal plasma cAMP levels, however, are normal in FHH patients. This excludes an important pathogenetic role for circulating cAMP in the granulocyte dysfunction (Marx *et al.*, 1980). It is not settled at the moment if local cAMP production is important in this syndrome.

We are aware that extrapolation of our *in vitro* results of granulocyte function to *in vivo* situation is hampered by the artificial test systems. Nevertheless, the functional impairment in the granulocytes of these patients might bear a direct correlation to the observed susceptibility to infections. The functional impairment might contribute to the overall poor prognosis of this familial condition. Indeed, recently two patients of this family died of respiratory insufficiency complicated by overwhelming pulmonary infection (among whom patient 1). Further studies using *in vivo* testing of host defence (e.g. skin window) might be desired to evaluate the precise pathogenesis of this familial condition.

Besides disturbances in granulocyte function the patients exhibited a significantly decreased IgM and IgG concentration in contrast to the hypergammaglobulinaemia which would be expected in view of their repetitive infectious episodes. The significant elevation of total haemolytic complement and C_1-esterase inhibitor are a non-specific expression of an inflammatory reaction. Total T-cells (E Rosette$^+$) and the balance between helper and suppressor cells were completely normal.

It was not possible to determine the pattern of inheritance of the increased liability for respiratory-tract infections in this limited number of patients who underwent investigation of granulocyte function. At present it is unclear to us whether any relationship exists between the FHH, the interstitial lung disease and the defective host defence. Although it may be tempting to suggest that the abnormal calcium metabolism is the common denominator, no arguments in favour of this hypothesis have been found. The three abnormalities did not necessary coexist in all patients (e.g. patient 3 and 5). Although different penetration is a possible explanation, it cannot be excluded that the coexistence may be coincidental.

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