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Defective Leukocyte Fungicidal Activity in End-Organ Resistance to 1,25-Dihydroxyvitamin D

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ABSTRACT. Recent studies have shown $1,25(OH)_2D_3$ receptor-mediated modulation of leukocyte proliferation, differentiation, and function. We examined the phagocytosis and killing of microorganisms by neutrophils and monocytes from five patients of three families with hereditary resistance to $1,25(OH)_2D_3$. Phagocytosis of microorganisms by patients' neutrophils and monocytes was normal. However, defective neutrophil killing activity toward *Candida albicans* (30–40% of controls) was found in all patients. The killing of *Staphylococcus aureus* was normal. The neutrophil chemiluminescence, nitroblue tetrazolium (NBT) dye reduction, and the generation of superoxide ions and hydrogen peroxide by neutrophils and monocytes after induction by either soluble stimuli or zymozan particles, did not differ from those in controls. The neutrophil myeloperoxidase activity was also normal. Monocytes obtained from two patients of different families before long-term calcium infusion therapy and after they became normocalcemic, demonstrated a similar impaired fungicidal activity toward *Saccharomyces cerevisiae*, indicating that hypocalcemia itself was not the cause of the killing defect. However, the addition of the Ca^{+2} ionophore A23187 (1 μM) to the test medium restored the monocyte fungicidal activity to normal. As patients' neutrophil cytosolic free calcium concentration was similar to that in controls, it is suggested that $1,25\text{-}(OH)_2D_3$ exerts its effect on leukocyte function by a putative receptor-mediated regulation of subcellular calcium localization which may be important for fungicidal activity. (*Pediatr Res* 25:276–279, 1989)

Abbreviations

$1,25(OH)_2D_3$, 1,25-dehydroxyvitamin D_3
TPA, 12-*O*-tetra-decanoyl-phorbol-13-acetate
f-met-leu-phe, N-formyl-L-methionyl-L-phenylalanine
NBT, nitroblue-tetrazolium

Recently, it has been shown that receptors for $1,25\text{-}(OH)_2D_3$, the hormonal form of vitamin D_3, are present in cellular components of the immune system and that specific functional receptors for $1,25\text{-}(OH)_2D_3$ mediate the effects of $1,25\text{-}(OH)_2D_3$ on the proliferation and differentiation of these cells (1–4). Previous observations have reported that vitamin D deficiency is associated with reduced neutrophil motility (5) and impaired phagocytosis (6). Moreover, impaired function of macrophages in vitamin D-deficient mice was corrected *in vitro* by $1,25\text{-}(OH)_2D_3$ (7). The occurrence of the syndrome of end-organ resistance to $1,25\text{-}(OH)_2D_3$ due to defective receptors or lack of biologic response to $1,25\text{-}(OH)_2D_3$ (8) provides an unusual opportunity of investigating the functions of $1,25\text{-}(OH)_2D_3$ in the immune system of the human subject. Indeed, peripheral blood mononuclear cells of patients with resistance to $1,25\text{-}(OH)_2D_3$ did not acquire receptors for $1,25\text{-}(OH)_2D_3$ after lectin stimulation as normal mononuclear cells do. Furthermore, in contrast to its effects on normal lymphocytes, $1,25\text{-}(OH)_2D_3$ failed to inhibit the proliferation of lectin-stimulated lymphocytes from patients with resistance to $1,25\text{-}(OH)_2D_3$ (9). In this study, we report our observations on the phagocytosis and intracellular killing of microorganisms by neutrophils and monocytes from five of the same group of patients.

PATIENTS AND METHODS

Five patients (four boys and one girl) aged 2 to 11 y of three families were studied. The patients had typical features of end-organ resistance to $1,25\text{-}(OH)_2D_3$ (10, 11) including rickets, alopecia, hypocalcemia, high serum $1,25\text{-}(OH)_2D_3$ levels and resistance to treatment with vitamin D_3 or $1,25\text{-}(OH)_2D_3$. Defective receptors for $1,25\text{-}(OH)_2D_3$ in cultured skin fibroblasts and lectin-stimulated peripheral blood mononuclear cells were demonstrated in all patients. Details of the receptor studies and patients' clinical and biochemical features are documented in previous reports (9, 11–13). Table 1 shows the main biochemical data at the time that the present tests were performed. Children admitted to the hospital for minor surgery served as controls. Informed consent was obtained from the parents of all children.

Neutrophils and mononuclear cells were isolated from heparinized venous blood by standard Ficoll-Hypaque gradients and dextran sedimentation techniques (14). Monocytes in the mononuclear cell fraction were allowed to adhere to plastic culture plates for 1 h in RPMI 1640 medium with 30% FCS. After washings to remove nonadherent cells, the adherent population was found to be greater than 95% monocytes as judged by the Wright-Giemsa stain. The phagocytosis and intracellular killing of *Candida albicans* and *Saccharomyces cerevisiae* were measured by the assay of Leherer and Cline (15), based on the differential staining of nonviable microorganisms by methylene blue. The phagocytosis and intracellular killing of *Staphylococcus aureus* were measured by the [^{3}H]uridine incorporation radio-

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Table 1. *Biochemical findings at time that present studies were performed*

Patients	Ca (mg/dl)	Pi (mg/dl)	Alkaline phosphatase (IU/ml)	$1,25(OH)_2D_3$ (pg/ml)
1	5.7–9.7*	3.2–4.6*	1600–350*	>600–88*
2	7.1–9.4*	3.8–4.5*	900–300*	>600–96*
3	6.5	4.3	990	280
4	7.0	3.9	1900	220
5	7.1	3.4	2000	180
Normal range	8.5–10.2	4.4–6	100–200	30–80

* Patient was studied before and during long-term calcium infusion therapy.

metric assay as previously described (16, 17). The assay depends upon the fact that external viable microorganisms incorporate [^{3}H]uridine, whereas those that are ingested by leukocytes do not. Briefly, 1×10^6 neutrophils were mixed with 1×10^7 *S. aureus* in 1 ml of RPMI 1640 medium with 5% normal human serum in small tubes and rotated end over end at 30 rpm at 37°C for 30 min. Controls lacking either *S. aureus* or neutrophils were also prepared. Phagocytosis was measured by taking a 100-μl aliquot from each tube to tubes containing 0.2 μCi of [^{3}H]uridine (Sp act 5 μCi/mmol Amersham Laboratories, Buckinghamshire, England) in 100 μl of medium. The tubes were then incubated at 37°C for 60 min. Intracellular killing was measured by taking a 100-μl aliquot from each tube into tubes containing 0.2 μCi [^{3}H]uridine, and the leukocytes were lysed in sodium deoxycholate (0.6%) plus DNAse (Sigma Chemicals Co., St. Louis, MO). The contents of the tubes were filtered through a membrane (Sartorius 0.45 μm, Sartorius Filters, Inc., Haywood, CA), washed three times, and dried for 2 h at 37°C. Associated radioactivity was measured in a liquid scintillation spectrometer. The percentage of ingested bacteria was calculated from the difference in the incorporation of [^{3}H]uridine into *S. aureus* in the presence or absence of leukocytes. The percentage of bacteria killed was calculated from the number of viable microorganisms added by lysis of the leukocytes to those not phagocytosed. This value was used to calculate the percentage of phagocytosed bacteria killed.

The endotoxin-stimulated NBT test was performed as described (18, 19).

The neutrophil Luminol-dependent chemiluminescence response to TPA (Sigma) was measured with a LKB-Wallac 1250 luminometer (LKB, Wallac, Finland). Leukocyte suspensions were prepared by hypotonic lysis of the red blood cells in 0.5-ml samples of heparinized blood. A leukocyte suspension of 100 μl (~ 5×10^6 cells) was placed into the luminometer in the presence of TPA (5×10^{-6} M) and 10^{-5} M Luminol (3-aminophthalhydrazide, Sigma). The results are expressed as light intensity in mV/ 5×10^6 cells. Production of superoxide (O_2^-) in neutrophils and monocytes was assayed by the superoxide dismutase inhibitable reduction of cytochrome C (20, 21). The neutrophils or monocytes were stimulated by either 100-nM TPA (Sigma) or by serum-opsonized zymozan particles (0.5 mg/ml, Sigma) for 60 min or by f-met-leu-phe (5×10^{-7} M, Sigma) for 5 min. The results are expressed as nmol O_2^- produced during the incubation time/10^6 cells. H_2O_2 production by polymorphonuclear leukocytes and monocytes was quantitated according to Pick and Keisari (22), using a method which is based on the H_2O_2-mediated and horseradish peroxidase-dependent oxidation of phenol red to a product whose absorbance is read at 610 nm. The results were expressed as nmol H_2O_2 produced by 10^6 cells in 60 min. The assay for myeloperoxidase activity involves following the change in absorbance at 450 nm that accompanies the oxidation of *O*-dianisidine (3.3′-dimethoxybenzidene; Sigma) (23). The results were expressed as nmol of dianisidine oxidized by 10^6 cells in 1 min.

The cytosolic free calcium concentration of neutrophils obtained from one patient was measured by using the fluorescent calcium indicator Quin-2 as previously described (24). In neutrophils from anothr patient, the free calcium concentration was measured using the calcium indicator fura-AM (Molecular Probs, Junction City, OR). Neutrophil (5×10^7/ml) cells were incubated with 5-μM fura-AM for 12 min and then diluted with 4 vol of buffer. After 30 min, the cells were pelleted, and fluorescence was measured in the 44B Perkin Elmer spectrofluorometer as described by Grynkiewicz *et al.* (25).

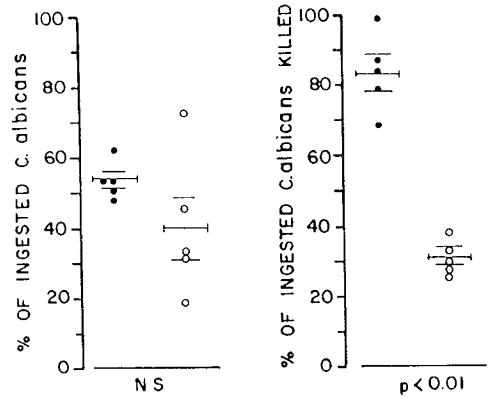

Fig. 1. Neutrophil phagocytic and killing activities towards *C. albicans* in five patients with end-organ resistance to $1,25(OH)_2D_3$ and in five controls. For experimental details, see "Patients and Methods." O, patients; ●, controls; ——, means ± SEM.

RESULTS

Fungicidal activity. The neutrophil candidacidal activity was significantly lower in patients with resistance to $1,25\text{-}(OH)_2D_3$ (31.3 ± 2.2% of ingested *C. albicans* was killed in 1 h) than in controls (82.8 ± 5.1%) (Fig. 1). Similarly, the ability of peripheral blood monocytes from two patients to kill *S. cerevisiae* was lower than in controls (Table 2). To rule out the possibility that hypocalcemia per se is the cause of the impaired killing of yeasts, the patients were studied again while on long-term intracaval calcium infusions and normocalcemic (26). The results showed that normocalcemia could not correct the impaired monocyte killing of yeast (Table 2). However, the presence of the calcium ionophore A 23187 (1 μM) in the test medium restored the monocyte-killing activity towards *S. cerevisiae* to normal (Table 2). A 23187 (1 μM) did not increase the killing of *S. cerevisiae* in monocytes from controls and had no direct effect on the viability of *S. cerevisiae* in the test medium.

Bactericidal activity. In contrast to their impaired fungicidal activity, the ability of the patients' neutrophils to kill ingested *S. aureus* did not differ from that found in controls (killing index, 107.6 ± 5.1% of controls).

Phagocytic activity. The neutrophil ingestion of *C. albicans* (Fig. 1) and the monocyte phagocytosis of *S. cerevisiae* (47.7 ± 3.8% of *S. cerevisiae* ingested in 1 h *versus* 51.4 ± 3.4% in controls) were comparable to those found in controls. Similarly, the ability of neutrophils to ingest *S. aureus* was normal (47.3 ± 2.5% *S. aureus* ingested in 1 h *versus* 44.6 ± 3.0% in controls).

The endotoxin-stimulated NBT test was normal in neutrophils of all patients (> 40% NBT-positive cells).

The chemiluminescence response of leukocytes from three patients to TPA stimualtion (58–78 mV) did not differ from the responses recorded in leukocytes of controls (54–98 mV). Incubation of neutrophils and monocytes obtained from one patient with either TPA or serum-opsonized zymozan resulted in the generation and release of superoxide, which did not differ from that observed in controls (Table 3). Similarly, neutrophils obtained from another patient that were stimulated by f-met-leu-phe generated 21 ± 0.8 nmol/10^6 cell/5 min of O_2^- as compared to 18.4 ± 0.8 nmol/10^6 cell/5 min in controls. The production

Table 2. *Monocyte killing activity in end-organ resistance to 1,25(OH)$_2$D$_3$ in two patients examined before and after intracaval Ca^{2+} infusion and in six controls in three to five experiments*

Treatment	Killing activity (% of ingested) *S. cerevisiae* killed in 1 h; mean ± SEM)		
	Patient 1	Patient 2	Controls
Hypocalcemia (no treatment)	19.8 ± 2.3*	21.3 ± 3.1*	47.2 ± 1.9
Normocalcemia (intravenous calcium)†	20.1 ± 2.4*	22.1 ± 2.8*	48.9 ± 2.0
Normocalcemia (intravenous calcium) + calcium ionophore—A 23187 (1 μM)§	48.5 ± 2.5‡	49.3 ± 3.0‡	48.7 ± 2.5
Normocalcemia (intravenous calcium) + A 23187 (0.1 μM)	36.3 ± 1.9*‡	35.7 ± 2.4*‡	48.7 ± 2.5

* Different from controls, $p < 0.01$.

† Normocalcemia was achieved in the patients following long-term intracaval calcium infusions. (See "Patients and Methods.")

‡ Different from patients' monocytes not treated with A 23187, $p < 0.01$.

§ The calcium ionophore was added to the killing test medium. (See "Patients and Methods.")

of H_2O_2 in neutrophils (38 ± 1 nmol/10^6 cells/60 min) and monocytes (13 ± 1 nmol/10^6 cells/60 min) after TPA stimulation, also did not differ from controls (39 ± 1 nmol/10^6 cells/60 min and 14 ± nmol/10^6 cells/60 min, respectively). The myeloperoxidase activity measured in neutrophils from two patients was 425 and 470 nmol of dianisidine oxidized/min in 10^6 cells. These results did not differ from those recorded in controls (460 ± 40 nmol/10^6 cells/min). The release of myeloperoxidase following f-met-leu-phe-induced degranulation of neutrophils from both patients and controls was 16–25% of the total myeloperoxidase activity.

The cytosolic free calcium concentration measured in neutrophils of one patient by using the fluorescent calcium indicator Quin-2 was 112 ± 9 nM as compared to 122 ± 14 nM in controls. In another patient, the free calcium concentration measured by the calcium indicator fura-2 AM was 160 nM as compared to 172–181 nM in controls.

Table 3. *Superoxide ion (O_2^-) production by neutrophils and monocytes in hereditary resistance to 1,25(OH)$_2$D$_3$*

	nmol/10^6 cells/60 min				
	TPA (100 nM) Neutrophils	Zymozan (0.5 mg/ml)		Control (unstimulated)	
		Monocytes	Neutrophils	Monocytes	Neutrophils
Patient 1	32 ± 4	35 ± 5	34 ± 4	13 ± 1	4 ± 0.3
Control 1	22 ± 3	45 ± 2	32 ± 2	15 ± 0	4 ± 0.3
Control 2	19 ± 2	35 ± 5	38 ± 2	12 ± 2	3 ± 0.2

DISCUSSION

The present study demonstrates an impaired leukocyte fungicidal activity in patients with end-organ resistance to 1,25-(OH)$_2$D$_3$. Our study indicates that only a fraction of the processes of intracellular killing of microorganisms is impaired. The adequacy of the remaining mechanisms is indicated by the normal bactericidal activity toward *S. aureus* and the fact that our patients are not troubled by recurrent serious infections. There could be an analogy with the condition of hereditary myeloperoxidase deficiency in which there is an impairment mainly in the candidacidal activity, which similarly does not cause a tendency to infection (27). However, the myeloperoxidase activity in neutrophils of patients with resistance to 1,25-(OH)$_2$D$_3$ did not differ from that recorded in controls. Production of superoxide and hydrogen peroxide by monocytes and neutrophils as well as the chemiluminescence responses of the patient's leukocytes to TPA were also comparable to results recorded in controls. These results suggest that an impairment in the oxidative burst mechanism is not the cause of the fungicidal defect in our patients.

The mechanism that links the observed defect of intracellular killing activity to the lack of normal receptors for 1,25-(OH)$_2$D$_3$, is not known at present. The absence of normal 1,25-(OH)$_2$D$_3$ receptors in the intestine results in impaired calcium absorption and hypocalcemia. However, hypocalcemia per se is probably not the cause of the impaired killing activity, as normalization of serum calcium levels could not correct the defect. It is reasonable to assume that the lack of 1,25-(OH)$_2$D$_3$ receptors in the leukocytes is linked to the observed intracellular killing defect. There are three likely explanations: The first is that since 1,25-(OH)$_2$D$_3$ may play a role in the differentiation of leukocyte precursors (28, 29), the cells of these patients may not be fully mature and lack a normal killing mechanism. The second possibility is that 1,25-(OH)$_2$D$_3$ regulates the synthesis of enzymes or other proteins which are concerned in the killing activity. The third possibility is that 1,25-(OH)$_2$D$_3$ affects the killing activity through its effect on the synthesis of proteins which regulate intracellular calcium metabolism. The observation that the calcium ionophore A 23187 restored the monocyte killing activity to normal makes the first two possibilities unlikely and suggests that the intracellular killing of yeast is dependent on normal redistribution of intracellular calcium, which is probably regulated by the 1,25-(OH)$_2$D$_3$ receptor-effector system. Confirmation of the latter hypothesis would require the identification of the putative 1,25-(OH)$_2$D$_3$-induced protein(s) and a demonstration of its importance to the killing process.

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Announcements

1989 Annual Meetings

The American Pediatric Society, the Society for Pediatric Research, and the Ambulatory Pediatric Association will have meetings on May 1–5, 1989 at the Washington Sheraton Hotel, Washington, D.C.

Contact: SPR—Ms. Debbie Wogenrich, 2650 Yale Blvd., S.E., Suite 104, Albuquerque, NM 87106 (505)764-9099. APS—Dr. Audrey K. Brown, Secretary-Treasurer, Department of Pediatrics, SUNY, Health Science Center at Brooklyn, 450 Clarkson Avenue, Box 49, Brooklyn. NY 11203 (718)270-1692. APA—Ambulatory Pediatric Association, 6728 Old McLean Village, McLean, VA 22101 (703)556-9222.

Annual Meeting of the European Society for Pediatric Research

The Annual Meeting of the European Society for Pediatric Research will be held in Kraków, Poland, June 11–14 1989. In addition to free paper and poster sessions, plenary sessions and workshops will be held on topics including acute renal failure, advances in genetics and immunology, applied genetics, perinatal epidemiology, neonatology, diabetes, and atrial natriuretic peptides. European Working Groups on Allergy and Clinical Immunooogy, Mineral Metabolism. Neonatogy and Perinatal and Pediatric Microcirculation will participate. *Deadline for receipt of abstracts will be January 31 1989.*

For abstract forms and information contact the President of the Meeting: Jacek J. Pietrzyk, 1st Department of Pediatrics. Institute of Pediatrics, 30-663 Kraków, Wielicka 265, Poland, phone 55-02-56, telex 032-5795 IP AM PL.