Periodic fever and mevalonate kinase deficiency

Periodieke koorts en mevalonaat kinase deficiëntie

(met een samenvatting in het Nederlands)

Proefschrift

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Door Joost Frenkel
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Mevalonate kinase deficiency and Dutch type periodic fever

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ABSTRACT

Dutch type periodic fever (DPF) is an autosomal recessive hereditary fever syndrome. Cases have been reported worldwide, the majority from France and The Netherlands. From infancy the patients suffer fever attacks that recur every 2-8 weeks, often precipitated by immunizations, infections or emotional stress. Fever lasts 2-7 days and can be accompanied by malaise, headache, diarrhea, abdominal pain, vomiting, skin rashes, arthralgia, arthritis,

tender lymphadenopathy, hepatosplenomegaly, and oral and genital ulcers. Labarotory evaluation during fever shows granulocytosis and elevated acute phase reactants. DPF is caused by a deficiency of the enzyme mevalonate kinase (MK). Besides DPF, the spectrum of MK deficiency includes a severe phenotype, mevalonic aciduria (MA). MA patients have less residual MK activity, leading to substantially higher urinary mevalonic acid excretion than in DPF. Mevalonic aciduria is characterized by mental retardation and dysmorphic features in addition to the clinical features of DPF. At the genomic level, several mutations of varying severity have been identified. The DPF phenotype is caused by one particular mild missense mutation. Most patients are compound heterozygotes for this mutation and a more severe mutation. The mechanism by which MK deficiency leads to fever is not understood. The vast majority of DPF patients have persistently elevated serum IgD and can be classified as having hyperimmunoglobulinemia D and periodic fever syndrome (HIDS). Conversely, most HIDS patients have MK deficiency and hence DPF, but the two disorders do not overlap entirely.

Introduction

The first reports of children with recurrent febrile attacks in the presence of an abnormally high serum IgD concentration were published in 1984 (1). The first detailed description of this disorder, was published by van der Meer *et a*l. (2), who introduced the term hyperimmunoglobulinemia D and periodic fever syndrome (HIDS). Most patients have been of Dutch extraction, although cases have been reported worldwide (3). Hence, the disease is also known as Dutch type periodic fever (DPF, MIM#260920). Inheritance is autosomal recessive. The first febrile crises usually occur in infancy and recur at varying intervals. The crises are typically triggered by infections and childhood immunizations. In addition to fever the patients often experience malaise, chills, headache, arthralgias, nausea, abdominal pain, and diarrhea, and show cutaneous rashes, hepatosplenomegaly, tender

cervical lymphadenopathy, and frank arthritis (3). Granulocytosis and elevated acute phase reactants during attacks reflect an acute inflammatory reaction, but a satisfactory explanation for these findings is lacking. Linkage analysis excluded the gene affected in FMF as a candidate involved in the etiology of HIDS (4). The cause of HIDS remained obscure until 1999. Then, HIDS patients were shown to have mutations in the gene *MVK*, which codes for the enzyme mevalonate kinase (MK, EC. 2.7.1.36) (5;6).

MK is an early enzyme in isoprenoid biosynthesis (Fig. 1). This biochemical pathway produces several compounds, including cholesterol. Deficient MK enzyme activity had been described previously in a rare autosomal recessive disease, mevalonic aciduria (MA, MIM# 251170) (7). MA is characterized by mild to severe mental retardation, cerebellar atrophy, manifested by progressive ataxia and dysarthria, myopathy, leading to hypotonia, and cataracts. Most of these children have dysmorphic facial features. Interestingly, MA patients suffer frequent inflammatory attacks, with fever, vomiting, diarrhea, arthralgias, cervical lymphadenopathy and hepatosplenomegaly, during which white blood cell counts and acute phase reactants are elevated (8) .Hence, despite the differences between MA and HIDS, the inflammatory crises are similar. The attacks are caused in some way by the MK deficiency, but it is not yet understood how. Not all patients with periodic fever and elevated IgD have MK deficiency (9;10). Most of what we know about DPF dates from before the identification of the molecular defect. Here we will use the term DPF for those patients who suffer recurrent febrile attacks and have reduced MK activity. The term HIDS then includes all patients with periodic fever and hyper IgD not explained by infectious or immunological diseases, irrespective of MK activity.

Epidemiology

At least 170 patients have been diagnosed with HIDS worldwide (11). Most of these are from Europe, the majority being Dutch (55%) and French (25%), with far smaller numbers being

reported from Sweden, Germany, the Czech Republic, the United Kingdom, Belgium, Italy, and Spain. Patients have also been reported from Turkey (12), Japan (13) and the United States (14). Heightened awareness of the disorder in France and The Netherlands may partially account for the higher numbers of patients reported from these countries as compared to neighboring states like Belgium and Germany. Though the exact incidence of HIDS is unknown, its prevalence in the Netherlands is estimated to be 5 or 6 cases per million total population. In reality, this figure is probably higher.

Symptoms

Disease onset in HIDS is, characteristically, in infancy. Subsequently, patients suffer repeated attacks, which can be precipitated by infections, minor trauma, physical or emotional stress, menses, and by childhood immunizations. Most attacks, however, occur without any clear precipitating event (3). The duration of the fever attacks can vary between patients and even from attack to attack in the same patient. In general, fever lasts for 2 to 7 days, more commonly 3 to 4 days. However, after the normalization of body temperature, it may take several days to weeks before general well-being is restored. Some patients with frequent febrile attacks do not recover entirely in-between. The episodes typically recur without strict periodicity, once every 3 to 6 weeks. However, patients may experience attacks from once every two weeks to once every 2 years. As patients grow older, attacks tend to become less severe and less frequent and there may even be prolonged disease-free intervals.

Fever is the dominant feature of the attacks. Body temperature rises abruptly, often with chills or rigors, remains high, often over 40°C, and then gradually returns to normal. Immediately preceding the attacks many patients experience malaise, headache, a sore throat or nasal congestion; in children irritability and hyperactivity are often noted. During the febrile attacks

the patients may have various other symptoms (Table I). Abdominal pain, diarrhea and vomiting are common. The pain is sometimes sufficiently severe to be confused with that of acute appendicitis. Non-bloody diarrhea is prominent in young children. Arthralgias accompany fever in most cases. Frank arthritis, mostly affecting the large joints of the lower extremities, is also common and may persist between crises. Patients frequently complain of severe headaches (3).

Signs

Tender cervical lymphadenopathy is a constant finding during febrile attacks (3) and the axillary, inguinal and intra-abdominal lymph nodes may also be swollen. Splenomegaly is present in nearly half of the patients, though it is a rare finding in adult patients, whereas hepatomegaly is almost exclusively seen in pediatric patients.Rashes have been observed in most HIDS cases. These may be macular, maculo-papular, urticarial, nodular or, rarely, purpuric (15). One Japanese patient has been reported with erythema elevatum diutinum (13). Oral and genital ulcers occur frequently in HIDS (16) and in one patient rectal ulcers have been observed (17). Serositis is not a regular feature of HIDS. However, acute intestinal obstruction due to adhesions has been described in 3 patients, 2 of whom had had no prior surgery (3).

Laboratory findings

Blood abnormalities during attacks are indicative of an acute phase response. Leukocytosis and granulocytosis are consistent findings; furthermore, the erythrocyte sedimentation rate and C reactive protein concentration are strongly elevated. In between attacks these values return to normal (18). Complement levels (C3, C4, CH50) are normal or raised with no indication of an increased consumption of complement, whereas circulating immune complexes are present at low levels in 20% of patients (3). About 16% of patients have microscopic hematuria during attacks, but impairment of renal function is very rare (19).

Immunological findings

IgD

IgD is a heavily glycosylated immunoglobulin. It comes in two forms, membrane bound and secreted. Its physiological role is largely unknown, as is its role in the pathogenesis of HIDS (20). In normal adults, serum IgD concentrations are below 100 IU/ml, but they can rise during infectious and non-infectious inflammatory diseases (21-23). Pediatric normal values depend on age. Infants have very little (<14.1 IU/ml) IgD, whereas normal schoolchildren may have serum IgD levels as high as 295 IU/ml (24).

Polyclonal elevation of serum IgD is a prerequisite for the diagnosis of HIDS. Hence, all patients reported have had raised serum IgD at some point in their disease course. There is, however, no relationship between disease activity and the IgD concentration (2). IgD can even be entirely normal at times, despite patients having active disease. In young children symptoms can exist for as long as 3 years before there

is any rise in serum IgD (25). With the recent establishment of normal values for serum IgD (24) it should be possible to identify HIDS earlier in preschool age children. However, in older children, there may be considerable overlap between these normal values and those observed in HIDS patients. With the identification of MK deficiency, it has become possible to recognize DPF in patients with persistently normal IgD levels. Only one such patient has been described, but this shows that increased IgD levels are not a prerequisite for the diagnosis of DPF (5). The high serum IgD level is more likely to be an epiphenomenon than a cause of the inflammatory state in HIDS, despite indications that IgD may stimulate cytokine secretion *in vitro* (26). Furthermore, elevated IgD is not unique for HIDS since it has been described in patients with other inflammatory conditions, notably the periodic fever aphtous stomatitis pharyngitis adenitis syndrome (23), familial Mediterranean fever (21;27), Behçet's disease (28), bronchiectasis, and immunodeficiency disorders (22), but it is not a consistent

finding in any of these disorders. The high serum IgD concentration in HIDS is due to increased production, as evidenced by the high numbers of IgD-containing plasma cells in bone marrow aspirates (2). The mechanism that leads to this increased synthesis of IgD in HIDS is unknown.

Other immunoglobulins

Other immunoglobulins are frequently elevated as well. Notably, IgA is raised in up to 93% of DPF patients, due to a rise in IgA_1 (29). Serum IgM can be high as well, but may be normal or even low. Similarily, serum IgG is usually normal but may be raised, predominantly due to elevated IgG3 levels.

Cytokines

Cytokine production in HIDS has been studied both *in vivo* and *in vitr*o, with attention focused on cytokines, known to induce fever and on mediators with anti-inflammatory properties. These measurements have been performed in plasma and in supernatants of cultured leukocytes from HIDS patients.

During febrile attacks the serum levels of interferon-γ and interleukin (IL)-6 rise sharply (30;31), and tumor necrosis factor (TNF)-α rises to high normal values, whereas IL-1α and IL-1β are not elevated (30). The effect of the increased stimulation of mononuclear phagocytes by interferon-γ is reflected in a rise in urinary neopterin excretion simultaneously with the onset of fever. The neopterin excretion remains high for several days after normalization of the body temperature (31). Serum levels of the anti-inflammatory mediators IL-1 receptor antagonist, soluble TNF receptor p55 and soluble TNF receptor p75 are raised during attacks, whereas IL-10 remains normal (30). The production of many of these mediators has been studied *in vitr*o. In supernatants of unstimulated cultures of whole blood samples, obtained during attacks, IL-1β is not increased (30). Culture supernatants of

peripheral blood mononuclear cells (MNC) obtained between attacks, however, contained high concentrations of IL-1β which increased even further upon stimulation with LPS (32) Although plasma levels of TNFα stay within normal limits, its concentration is increased in the supernatants of unstimulated cultures of whole blood samples drawn between attacks. When stimulated with LPS, the supernatants of such cultures showed an elevated TNFα level, which was even higher when the blood cells had been obtained during an attack (30). Similar results were obtained in culture supernatants of isolated MNC obtained between attacks (32). Notably, spontaneous IL-1β, IL-6 and TNFα production by isolated MNC is elevated in HIDS and rises further on stimulation with LPS. Taken together these data are compatible with macrophage activation during the febrile attacks. Between the febrile attacks the *in vitro* findings are still compatible with an increased activity of the mononuclear phagocytic compartment. The cause of this macrophage activation is unknown. While activated Thelper-1 cells are known to induce macrophage activation, the high IgD and IgA levels are more compatible with an increased activity of Thelper-2 cells (33). However, direct evidence of T-cell activation during attacks is lacking.

Biochemistry

The identification of MK deficiency as the cause of DPF links this syndrome to mevalonic aciduria. This latter inborn error was first described by Berger *et al.* in 1985 (34) and subsequently recognized to be caused by a deficiency of MK enzyme activity (7). In MA the MK enzyme activity is usually virtually absent when measured in cultured skin fibroblasts or lymphoblasts of patients (<4% of the control mean, usually<0.5%)(8;35). In DPF, however, a residual MK activity varying between 1 and 8% can be measured both in fibroblasts and leukocytes from patients (5;36). As a result of the MK deficiency, excretion of mevalonic acid in urine occurs, although the levels of excreted mevalonic acid vary significantly between both syndromes. MA is characterized by a massive and constitutive excretion (1000-56000

mmol/mol creatinine) (8), while in DPF the mevalonic acid excretion is moderate (4-28 mmol/mol creatinine) and may be normal between febrile crises (5) In healthy controls, the excretion of mevalonic acid in urine is usually less than 1 mmol/mol creatinine.

MK is the first enzyme to follow 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) in the mevalonate pathway and converts mevalonate into 5-phosphomevalonate (Fig. 1). The mevalonate pathway provides cells with isoprenoids that are essential for diverse cellular processes. The main end products include ubiquinone-10 and haem A, both of which are necessary for electron transport, isopentenyl tRNA (involved in protein translation), dolichol (essential for N-linked protein glycosylation), and the farnesyl and geranylgeranyl groups used for isoprenylation of the proteins involved in cell proliferation and differentiation. In addition to these non-sterol isoprenoids the mevalonate pathway produces cholesterol, a structural component of cellular membranes and precursor for bile acids and steroid hormones (37). HMG-CoA reductase, which converts HMG-CoA into mevalonate, catalyzes the main rate-limiting step in isoprenoid biosynthesis and is among the most tightly regulated enzymes in nature (37). Indeed the statins, which are drugs widely used to treat atherosclerosis and familial hypercholesterolaemia, are potent competitive inhibitors of the reductase; they block the synthesis of mevalonate and as a consequence lower the endogenous synthesis of cholesterol. The use of statins can trigger adaptive reactions yielding up to 200-fold increased HMG-CoA reductase activity. A therapeutic trial in which two MA patients were treated with low doses of lovastatin in order to block mevalonate production, however, was unsuccessful and had to be abandoned because of the development of severe clinical crises (8).

Several metabolic diseases have been described which are caused by defects in the isoprenoid pathway. Five disorders, Smith-Lemli-Opitz syndrome (38-40), desmosterolosis (41), CHILD syndrome (42), Greenberg dysplasia (43) and X-linked dominant chondrodysplasia punctata

(44;45)only affect the sterol biosynthesis. MA and DPF affect the biosynthesis of all isoprenoids. Periodic fevers and dysregulation of the immune system are only present in DPF and MA, which indicate that a shortage of cholesterol is not the pathogenetic basis of the periodic fevers. This is also supported by the observation that cholesterol levels are usually (near) normal in patients with MA (8). Furthermore, the incorporation of radiolabeled acetyl-CoA into cholesterol by cultured skin fibroblasts of DPF and MA patients appears to be comparable with that in controls (7).The differences in mevalonate excretion between DPF and MA could be caused by a difference in the regulation of HMG-CoA reductase activity. In MA there may be a constitutive induction of HMG-CoA reductase, as suggested by the elevated reductase activity in fibroblasts from MA patients and the levels (7) of excreted mevalonate (> 800 mmol/ day) which greatly exceed the level of normal whole body cholestrol biosynthesis (4 mmol/day, equivalent to 24 mmol of mevalonate/day) (8), while in DPF the reductase activity appears only to be raised during fever episodes(46)

Molecular biology

The cause of DPF was independently identified in 1999 by a Dutch research group and Dutch/French consortium using two different approaches. Both groups ended up with the same gene, *MV*K, coding for the enzyme MK. Houten *et a*l. (5) performed organic acid analysis of urine from one periodic fever patient during

an episode of fever and found elevated levels of mevalonic acid. This patient had all the characteristics of HIDS except for an elevated IgD. Subsequent organic acid analysis in 2 HIDS patients, however, also revealed elevated levels of mevalonic acid during episodes of fever. The elevated levels of mevalonic acid in urine suggested a defect in the metabolism of mevalonate, and enzyme measurements revealed a profound deficiency of MK enzyme activity. Subsequent mutation analysis revealed several disease-causing mutations in the

MVK gene. Drenth *et a*l. performed linkage analysis and obtained linkage at chromosome 12 position q24 (6). After narrowing the region to 9 cM, they concluded that *MVK* was a good candidate gene and subsequently identified mutations in this gene in several HIDS patients.Since the human MVK cDNA sequence had already been cloned, both groups performed mutation analysis at the cDNA level. This revealed a common missense mutation, a G > A transition at nucleotide 1129 changing the valine at position 377 into a isoleucine (V377I), in 32 out of 38 HIDS patients (10;36;47). Most patients were compound heterozygotes for this mutation. Two additional missense mutation were commonly identified. an A > C transversion at nucleotide 59 and a T > C transition at nucleotide 803, which change the histidine at position 20 into a proline (H20P) and the isoleucine at position 268 into a threonine (I268T), respectively. Since the latter two mutations were also identified in MA patients (35;48), this strongly suggested that the V377I mutation is responsible for the DPF phenotype of MK deficiency. Indeed, thus far only 6 patients have been described without this mutation (10;36). One of these was compound heterozygous for a C > T transition at nucleotide 500, changing the proline at position 167 into a leucine (P167L). The other allele carried the I268T mutation, which was also found in MA patients, suggesting that the P165L mutation is responsible for the DPF phenotype in this patient.

The *MVK* gene was previously reported to be a single copy gene located on chromosome 12 postion q24 (49;50), which is in accordance with the linkage analysis performed by Drenth *et a*l. (6). We have recently resolved the genomic organization of the mevalonate kinase gene, showing it to consist of 11 exons (36). Figure 2 is a schematic representation of the *MVK* gene and includes all the mutations currently identified in both MA and Dutch type periodic fever.

In order to study the effect of the identified missense mutations on MK activity, most of the mutant proteins have been characterized by immunoblotting and heterologous expression. The

first identified mutation in MA, N301T, was expressed in COS-7 cells and showed a markedly decreased enzyme activity varying between 5 and 20% of the activity of wild type MK (49). More recently, a mutation in MA was characterized, which changed the affinity of the enzyme for its substrate mevalonate when expressed as a recombinant fusion protein in *Escherichia coli* (A334T) (51). The V377I mutation had considerable residual activity when expressed in *E. coli* (35% compared to the control), but MK protein in fibroblast lysates of HIDS patients was hardly detectable, as shown by immunoblotting with an MK-specific antibody. This indicates that, although this mutation has some effect on enzyme activity, it predominantly affects the stability of the MK protein *in vivo*. (5). All of the other characterized mutations result in MK proteins with markedly decreased enzyme activity when expressed in *E. coli* and/or decreased protein levels in fibroblast lysates (H20P, T243I, L264F, L265P, I268T and V310M) (35;48).

Differential diagnosis

DPF should be distinguished from a number of hereditary and non-hereditary disorders with which it has features in common. First, infectious diseases and cyclic neutropenia should be ruled out. Systemic onset juvenile chronic arthriis, like DPF, is characterized by fevers, transient rashes, lymphadenopathy, hepatosplenomegaly, and arthritis. However, the onset is rarely in the first year of life, the fever has a spiking pattern, the disease flares usually last many weeks instead of days, and the arthritis is destructive. Contrary to DPF, serositis is common and diarrhea is usually absent. Furthermore, there is no ethnic predisposition and the disease is not familial in occurrence. Familial Mediterranean fever typically occurs in patients of Turkish, Armenian, Iraqi or Sephardic Jewish ancestry. It is characterized by recurrent bouts of high fever, accompanied by arthritis and by severe abdominal pain due to serositis. Patients may have pleuritis, pericarditis, acute scrotal pain or an erysipelas-like rash of the

lower part of the legs (52-54). The onset of attacks is usually at a slightly later age (2-10 years) than in HIDS and the attacks typically last only 12-72 hours (55). Diarrhea, headache, generalized rashes, lymphadenopathy and hepatosplenomegaly do not feature in FMF. Amyloidosis is a common serious late complication of FMF, which may lead to nephrotic syndrome and renal failure. In contrast to DPF, the attacks of FMF can be largely prevented by colchicines (56). This treatment reduces the future development of amyloidosis. Inheritance is autosomal recessive and the responsible gene, *MEF*V, on chromosome 16p13.3 codes for a protein of unknown function called pyrin or marenostrin (57;58). Molecular diagnosis is feasible in most FMF patients, but in at least 20% of patients one or both alleles appear normal (53;59-62). FMF therefore remains primarily a clinical diagnosis (63)

The TNF-receptor associated periodic syndrome (TRAPS) is a rare disorder that has been encountered primarily in Irish and British families and was therefore called familial hibernian fever, but it has also been encountered in families from other countries, including France, Finland, Israel, and The Netherlands. The onset of this disease is between 2 and 20 years of age, after which febrile attacks recur at irregular intervals, typically 2 to 4 times every year (64). The duration of fever varies from some days to many weeks. The fever may be accompanied by edema of the eyelids, conjunctivitis, myalgias, painful erythema of the arms and legs, arthralgias, and scrotal and abdominal pain. As in FMF, these patients may have serositis, and some have developed amyloidosis. In contrast to DPF, lymphadenopathy is absent. Inheritance is autosomal dominant and molecular diagnosis has become possible with the identification of the affected gene, *TNFR1A*. This gene encodes the 55kDa TNF receptor protein (65) and mutations affect the extracellular domain of this receptor, interfering with receptor shedding.

Familial Cold-induced Autoinflammatory syndromeand Muckle Wells syndrome.

These two rare syndromes with autosomal dominant inheritance were shown in 2001 to be due to mutations in the same gene. This gene, *CIAS1*, encodes cryopyin, a protein related to pyrin / marenostrin (66). Affected patients have recurrent short-lived (<24 hours) episodes of fever and urticariform rash from infancy. Cojunctivitis and arthralgias are (67) often present during the attacks. Attacks triggered by exposure to cold define the clinical phenotype of cold-induced autoinflammatory syndrome, whereas perceptive deafness and amyloidosis characterize the Muckle-Wells syndrome. In fact, there is overlap between these phenotypes, even within affected families (68).

The CINCA syndrome, which stands for Chronic Infantile neurologic cutaneous and articular syndrome is also known as Neonatal onset multi-system inflammatory disease (NOMID) (1;69;70).It is a sporadically occurring inflammatory disease with recurrent short fever bouts, non-pruritic urticara, deforming arthropathy. Neurological involvement consists of aseptic meningitis, intracranial hypertension and cerebral atrophy ad is reflected in headache, varying degrees of developmental delay, focal deficits and seizures. Patients often fail to thrive and remain short statured. Uveitis and papillitis optica may lead to blindness. Peceptive deafness is a common sequel. Dysmorphic features, such as frontal bossing and saddle nose, develop gradually. CINCA is a clinical diagnosis.

The periodic fever aphtous stomatitis, pharyngitis and adenopathy (PFAPA) syndrome is characterized by febrile attacks that last 2-8 days and recur every 2-8 weeks (23;71;72). The fever is accompanied by aphthous stomatitis, cervical lymphadenopathy and pharyngitis, but some patients also experience headache, abdominal pain, diarrhea or rashes. IgD may be mildly elevated. Hence, differentiation from HIDS can be particularly difficult. The PFAPA syndrome is, however not familial in occurrence (23;72).

Although the majority of patients initially classified as HIDS were MK deficient when tested, about 25% of patients were found to have normal MK activity. These children tend to have a

later age of disease onset, but could not otherwise be distinguished from DPF cases (9). These patients have been said to have variant HIDS (73). Ultimately, the correct diagnosis of HIDS sensu strictu, i.e. DPF, can only be made by the demonstration of a reduced MK activity in cultured skin fibroblasts or mononuclear cells or by the demonstration of mutations in both alleles of the *MVK*-gene.

There is no uniform, evidence based diagnostic approach for children with recurrent fever. The overlapping clinical presentations defy a straightforward decision tree. Rather, the combination of clinical and epidemiological features in a given patient makes certain diagnosis less or more probable. In table 2 such features of the various periodic fever disorders are set side by side. The probability for a given feature to point to a specific diagnosis is, however, qualitative at best. To enable a more quantitative approach, prospective studies are needed in children, who present with recurrent fever of unknown origin,. A European working party (www.pres.org.uk) is currently addressing this question.

Treatment

Most therapeutic interventions in HIDS have not been systematically studied. Non-steroidal anti-inflammatory drugs, colchicine, intravenous immunoglobulins, and cyclosporin A have been tried in some patients and were, in most cases, found to be ineffective. Some patients responded favorably to corticosteroids. The long-term use of corticosteroids in childhood has too many side effects, however, to justify their use as maintenance treatment to prevent attacks. Thalidomide was tested in a double-blind placebo-controlled trial (74). There was no significant clinical benfit.

Prognosis

The frequency and the severity of attacks appear to decrease with age. In our experience, however, all children with DPF have remained symptomatic. In contrast, children with variant HIDS, i.e. recurrent fever and elevated serum IgD but normal MK activity, were likely to

enter long-term remission. There are some indications that pregnancy may further decrease disease activity (75). Some patients have long-term sequelae, however. Three patients developed peritoneal adhesions, possibly as a consequence of serositis. One child, diagnosed with HIDS, developed end stage renal failure due to crescentic glomerulonephritis (76).That patient was, however, later shown to have MA (19). This shows the two disorders are really part of a continuous spectrum of mevalonate kinase deficiency. Joint damage has not been reported in HIDS.

Among the patients in the HIDS registry two deaths have been reported, one by suicide and one from a stroke (3). It is doubtful whether these complications are related to HIDS. Though HIDS may cause little excess mortality or permanent organ damage, the impact of a disease that causes patients to miss school frequently from kindergarden through high school cannot be underestimated. Data on the psychosocial effects are lacking, however.

Future directions

The identification of the molecular defect in DPF will have implications for our understanding of the disease, its pathogenesis, its natural history and its treatment. Efforts are underway to elucidate the pathophysiological mechanisms by which mevalonate kinase deficiency leads to periodic fever. Other defects in the isoprenoid pathway with similar clinical effects might be expected, but these have not yet been identified.

With a definitive diagnostic criterion at hand, the patient population is better defined, which will enable us to reappraise the clinical presentation and the natural course of the disease. Finally, we are now in a position to assess prospectively the effect of interventions in a circumscript patient group. The involvement of the isoprenoid pathway in the pathogenesis suggests that treatment with statins, which interfere in the earliest step of this route, might be beneficial. A clinical trial with such a compound is underway in adults

(11).Results of both mechanistic studies and intervention trials should ultimately shed light on the physiological role of isoprenoid metabolism in inflammation

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Table I. Cumulative incidence of symptoms and signs during febrile attacks in HIDS (modified from reference 3).

Fever 100%	Lymphadenopathy 94%
Chills 76%	Skin rash 82%
Abdominal pain 72%	Splenomegaly 48%
Diarrhea 82%	Serositis 6%
Vomiting 56%	Arthritis 68%
Headache 52%	
Arthralgias 80%	

Table 2: Comparison of Childhood Autoinflammatory Diseases

	FMF	HIDS (DPF)	TRAPS	FCAS / MWS	CINCA	Systemic JIA	PFAPA
Inheritance	AR	AR	AD	AD	Unknown	None	None
Gene	*MEF-V*	*MVK*	*TNFRSF1A*	*CIASI*			
Protein	Myrenostrin (Pyrin)	Mevalonate Kinase	TNFRSF1A	Cryopyrin			
Function	Unknown	Isoprenoid biosynthesis	TNF receptor	Unknown			
Usual ethnic origin	Jews, Arabs, Turks, Armenians	Dutch, French, Other	British, Irish, Other	European	Worlwide	Worldwide	Worldwide
Usual onset age	Childhood	Infancy	Variable	Neonatal or infancy	Neonatal	Childhood	Pre-school
Duration of Attacks	12 – 72 hrs	3 – 5 days	Days to weeks	< 24 hrs	Several hours	Weeks	3 – 6 weeks
Interval between attacks	Weeks to Months	Weeks to Months	Weeks to months	Variable	Days	Weeks or none	3 – 6 weeks
Vomiting		+					+
Diarrhea		+	+				+
Abdominal pain	++	+	+			+	+
Peritonitis	++		+			+	
Pleuritis	+		++			+	
Scrotal pain	Infrequent		++				
Rashes	Erisipelas-like (rare)	++ (Various)	++ Migratory tender erythematous plaques	++Urticaria	++ Non-pruritic urticaria	++ Evanescent pink macules	
Eye disease			++ conjunctivitis, periorbital oedema	Conjunctivitis	Papillitis, uveitis		
Hearing				MWS: deafness	Perceptive deafness		
Mucosal Involvement		Aphthous ulcers					++ Aphthous ulcers, pharyngitis
Joint Involvement	Monoarthritis	Arthralgias, oligoarthritis	Arthralgias	Arthralgias	Destructive arthritis of large joints	Destructive Polyarthritis	Arthralgias (rare)
Headache		+	++	+	+		+
Myalgias	Infrequent	Infrequent	++	+		+	
Lymphadenopathy		++	+		+	+	++
Splenomegaly		+	+		+	+	
Amyloidosis	+		+	FCAS + MWS ++	+	+	
Helpful tests	Response to colchicine	Elevated serum IgD, IgA, mevalonic aciduria acid	Serum soluble TNFRSF1A		CSF Pleiocytosis		
Diagnostic confirmation	*MEFV* gene analysis	*MVK* gene analysis	*TNFRSF1A* gene analysis				

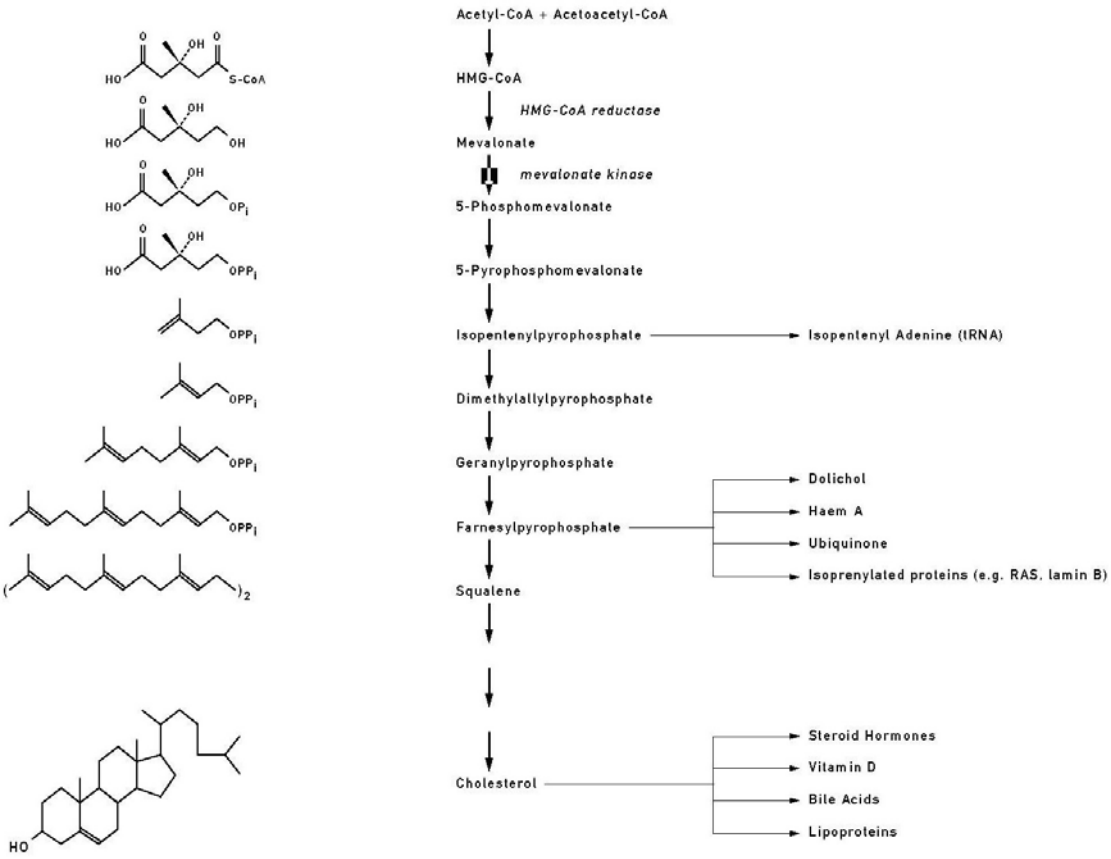

Fig. 1. Isoprenoid biosynthesis. Mevalonate kinase catalyzes the reaction immediately after HMG-CoA reductase. End products of this route include isopentenyl tRNAs, dolichol, Heme A, ubiquinone, farnesyl and geranylgeranyl groups for protein isoprenylation, and cholesterol, with all its derivatives. In mevalonate kinase deficiency there is accumulation of mevalonic acid.

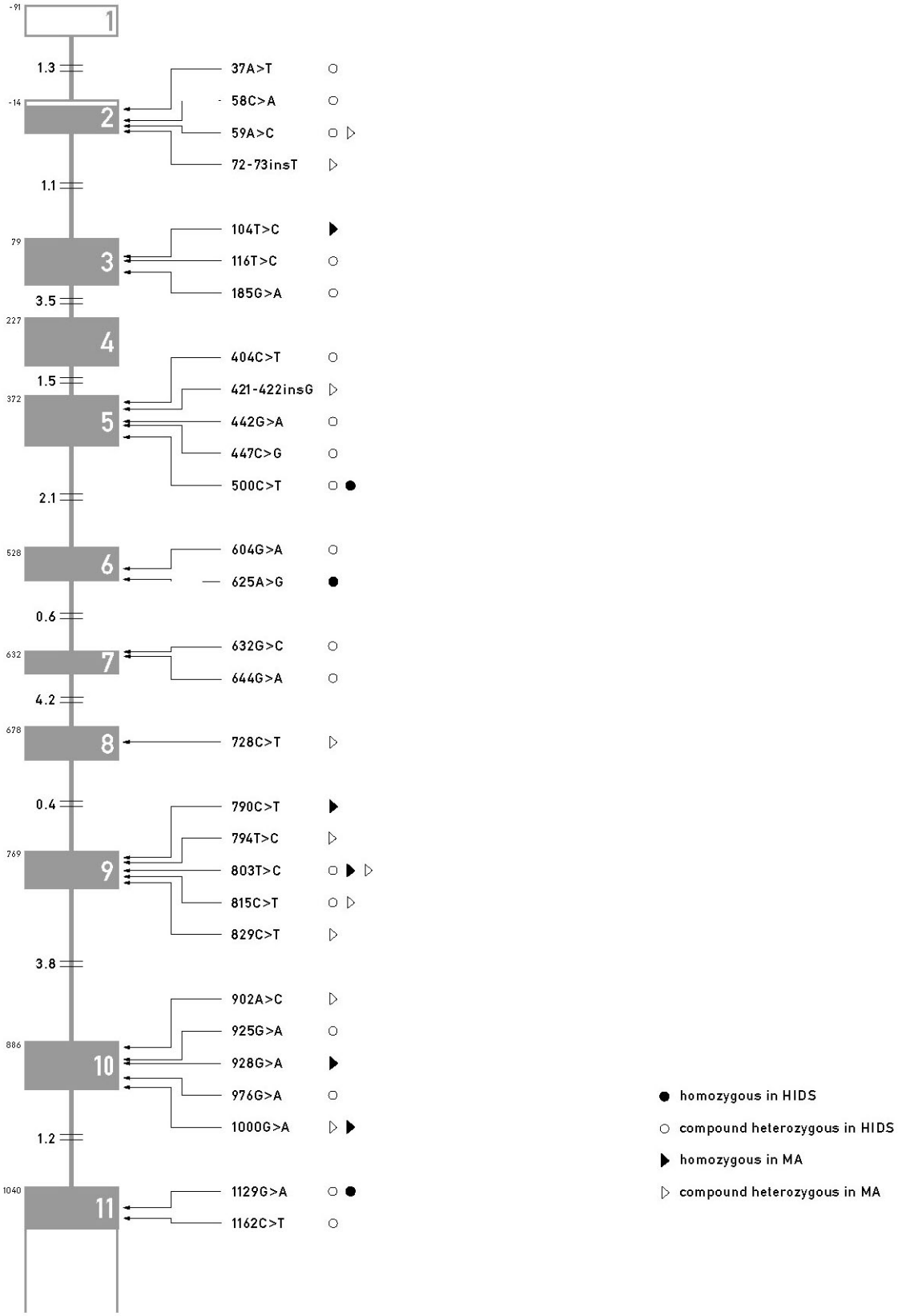
-91
1
1.3
37A>T
-14
58C>A
2
59A>C
72-73insT
1.1
104T>C
79
3
116T>C
185G>A
3.5
227
4
404C>T
1.5
421-422insG
372
5
442G>A
447C>G
500C>T
2.1
528
6
604G>A
625A>G
0.6
632
7
632G>C
644G>A
4.2
678
8
728C>T
0.4
790C>T
794T>C
769
9
803T>C
815C>T
829C>T
3.8
902A>C
925G>A
886
10
928G>A
976G>A
1000G>A
1.2
1040
11
1129G>A
1162C>T
homozygous in HIDS
compound heterozygous in HIDS
homozygous in MA
compound heterozygous in MA

Fig. 2. Schematic representation of the organization of the *MVK* gene. The locations of all the mutations identified at present in Dutch type periodic fever and mevalonic aciduria are indicated.

Clinical and molecular variability in childhood periodic fever with hyperimmunoglobulinaemia D

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Running title: Variability in Hyper IgD syndrome

Abstract

Objectives

The hyperimmunoglobulinemia D and periodic fever syndrome (HIDS) was recently found to be caused by a deficiency of mevalonate kinase (MK). The aim of this study was to examine, whether a relationship exists between the clinical expression of HIDS and the extent of MK deficiency.

Methods

Medical records of children diagnosed with HIDS were reviewed for clinical features and serum immunoglobulin values. The mevalonic acid excretion in urine and the enzyme activity of MK in patient's cells were measured and the *MVK* cDNA's sequenced

Results

Fifteen patients with recurrent fever and raised serum IgD were included. Their clinical features varied. Eleven patients had a deficiency of MK, caused by mutations in the *MVK* gene. One mutation (V377I) was common to all eleven patients. Nine patients were compound heterozygous for V377I and various other *MVK* mutations. There was no apparent relation between the observed mutations and the clinical features. Surprisingly, four boys had normal MK activity and no *MVK* mutations

Conclusions

Most HIDS patients have mutations in the *MVK* gene. The clinical variability observed can not be explained by genotypic differences. Periodic fever and elevated IgD can result from other, still unknown causes. Hence testing for MK deficiency is necessary in patients with unexplained periodic fever.

Abbreviations

HIDS	Hyper IgD Syndrome
MK	Mevalonate kinase
MNC	Mononuclear cells
MVK	Gene encoding mevalonate kinase
FMF	Familial Mediterranean Fever
PFAPA	Periodic Fever Aphtous Stomatitis Pharyngitis Adenitis

Introduction

The hyperimmunoglobulinemia D and periodic fever syndrome (HIDS), also known as Dutch type periodic fever (MIM#260920), is an autosomal recessive disorder, characterised by febrile attacks recurring at more or less regular intervals and the presence of an elevated serum IgD concentration (>100 IU/ml) (1). Clinical features of the febrile attacks include cervical lymphadenopathy, splenomegaly, hepatomegaly, skin rash, vomiting, diarrhoea, arthralgias, and arthritis. Patients often complain of malaise, chills, headache, nausea or abdominal pain (2). The diagnosis of HIDS has always been based on the occurrence of both recurrent unexplained fever and elevated serum IgD levels. Recently, we and others identified the underlying genetic defect of the syndrome, namely mutations in the gene *MVK*, which lead to a deficiency of the enzyme mevalonate kinase (3;4). MK catalyses the phosphorylation of mevalonic acid into 5-phosphomevalonate. It is the enzyme immediately following 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase in the isoprenoid biosynthesis pathway. This pathway produces cholesterol and numerous non-sterol isoprenoids, involved in a large variety of cellular processes (5). In HIDS, the deficiency of MK becomes evident during febrile attacks, when its substrate, mevalonic acid, accumulates in plasma and is excreted in the urine. Although it is known that febrile episodes in HIDS can be triggered by injuries, infections, or vaccinations, it is unclear how these exogenous factors interact with the endogenous biochemical defect to give rise to the inflammatory episodes. So far, 9 cases of HIDS have been published in whom MK activity had been determined (3;4). All were found to have a markedly reduced MK enzyme activity. Here, we describe our findings in a group of 15 children with HIDS, including 4 who have been previously described (2;3). Surprisingly, in 4 children normal MK activity was found. These cases will be described in more detail.

Patients and methods

We included in this study all children with HIDS, who had been visiting the 3 participating children's hospitals between January 1997 and October 1999. The diagnosis of HIDS was based on the following criteria: 1) recurrent fever over 38.5°C, not explained by infections or otherwise and 2) a serum-IgD >100 IU/ml established at least once, preferably on 2 occasions at least 1 month apart. Throughout the text patients are identified by their entry number in the international Hyper IgD study group registry (2). Four patients (numbers 26, 50, 132, and 139) have been described previously (2;3).

After approval by the medical ethical committee and informed consent by parents, patient charts were reviewed for clinical characteristics, disease history, and serum immunoglobulin levels.

MK activity was determined in peripheral blood MNC using ^{14}C-labelled mevalonate as substrate (6)speidel and expressed as the percentage of the average MK activity, determined simultaneously in MNC from healthy control subjects. Normal values are 166.3 ± 69.1 nmol/min.mg.

Urine samples were collected during febrile crises, not due to intercurrent infection.

Mevalonic acid was measured in, by stable isotope dilution gas chromatography mass spectroscopy of the trimethylsilyl ether / esters using $^{2}H_{7}$ –mevalonolactone as an internal standard (7).

In all patients, mutation analysis of the *MVK* cDNA was performed as described previously (8). Briefly, cDNA was prepared from total RNA isolated from MNC. Two sets of *MVK*-specific primers with –21M13 or M13rev extensions were used to amplify *MVK* cDNA in 2 overlapping fragments by PCR. Both PCR fragments were sequenced in both directions using

fluorescent primers on an ABI 377A DNA sequencer (Perkin-Elmer), according to the manufacturer's instructions.

Results

Clinical heterogeneity

The demographic data and disease courses of the 15 patients are summarised in table 1. Of the 15 patients 5 were girls and 10 were boys. Age ranged from 2 to 20 years with a mean age of 9.4 years. One patient (No 138) was of Turkish descent, whereas the remaining 14 patients were of Dutch origin. There was no consanguinity in any of the families and there were no additional family members affected. The febrile attacks started between 2 months and 14 years of age, with a median of 6 months. On average, the fevers lasted for 4.2 days and recurred every 3 to 7 weeks. In individual patients, however, the duration of the febrile episodes varied considerably. Similarly, the interval between attacks varied during the disease course from less than 2 weeks up to many months. In fact, 2 children (No 50 and 138) had been free of symptoms for over 12 months.

Table 1 summarises the clinical features accompanying the febrile crises. All children had fevers up to 40°C, accompanied by malaise and fatigue. In many instances, fever was elicited by mental stress, strenuous exercise or infections. Pharyngitis was documented in 8 patients, pneumonia in 7, Otitis in 5, but there were no recurrent severe infections in any patient. Childhood vaccinations were followed by fever in the majority of patients. However, most attacks came apparently unprovoked.

Usually there were prodroma such as fatigue, anorexia or behavioural changes. Onset of fever was brisk, with 40% of patients experiencing chills. One patient (No139) suffered repeated febrile seizures. Temperature normalised after 3 to 7 days of high fever. Subsequently, most patients remained listless and tired for another 3 to 7 days.

The rashes that were reported in 11 of 15 patients varied from faint erythema to maculopapular and urticarial exanthemata. One patient had recurrent swelling of hands and feet during attacks, another had so only once. Of the 15 patients, 11 reported headache during attacks. Abdominal pain was very common, often accompanied by diarrhoea and/or vomiting. Arthralgias were present in 80% of patients, whereas arthritis, defined as painful swelling of joints was rare. Lymphadenopathy, hepatomegaly, and splenomegaly were present in the majority of patients. Notably, there was no recurrent pharyngitis or aphtous stomatitis in any patient.

Growth was normal in all children, but 2 patients had very mild developmental delay and 1 patient (No136) was severely mentally retarded . He died at the age of 17 years.

Biochemical and molecular heterogeneity

Serum IgD levels were raised above the upper limit of normal (100 IU/ml) at least once in all 15 children and on more than one occasion in 14. Serum levels of IgG, A, and M were repeatedly raised in most patients (table2).

Eleven of 15 patients were found to have a deficient mevalonate kinase activity in peripheral blood MNC (1–7%, normal 100 ± 42%), In 9 of these 11 patients urine was obtained during fever. Urinary mevalonic acid concentration, normally below 1 mmol/mol creatinine, was elevated in all 9 samples (4.8 - 41.5, average 17.5 mmol/mol creatinine), whereas it was not elevated in the three living patients with normal MK activity.

In all 11 patients with reduced MK activity mutations could be identified in the *MVK* cDNA. The observed mutations and their consequent amino acid changes are represented in table 3. All MK deficient patients carried the substitution of the guanine at position 1129 by an adenine (1129G→A), leading to an amino acid change from valine to isoleucine at position 377 (V377I). Two patients were homozygous for this mutation at the cDNA level, confirmed

by sequencing of the parental cDNA's. All other patients were compound heterozygous for 2 mutations (table 3).

Clinical features appear unrelated to molecular variability

We found no clear relation between the different genotypes and residual MK activity. Neither the genotype nor the degree of enzyme deficiency correlated with the clinical severity of the disease. In 4 male patients a normal MK activity was found (No's 50,138, 141, and 143). These boys will be described in more detail below.

Normal MK activity in four patients

Case No 50: This 8-year old Dutch boy had suffered repeated bouts of fever from the age of 5 weeks. Fever started abruptly and remained high for 2 to 5 days, recurring every 2 to 3 weeks. Episodes were often triggered by minor infections. General malaise and painful cervical lymphadenopathy accompanied the attacks. On occasions he suffered from a sore throat. He had no rash, oral ulcers, arthralgias, headache, abdominal pain, diarrhoea or vomiting during attacks. Once, mild hepatomegaly, without splenomegaly, was noted.

During attacks, there was leukocytosis (14.1-16.7x10 ^{9}/l) with granulocytosis and band forms. Paracetamol reduced the temperature, but non-steroidal anti-inflammatory agents were ineffective in preventing attacks.

Case No 136: This 17-year old Dutch boy was born small for date (2250g) after a full term pregnancy. He was noted to have hypotelorism, antimongoloid, slanting short palpebral fissures, low-set ears, high arched palate, dental crowding, and clinodactyly of the fifth fingers. His psychomotor development was severely delayed. No syndromal diagnosis had been made, despite full metabolic and cytogenetic workup.

From the age of 4 years, he had suffered repeated attacks of diarrhoea.

Recurrent bouts of fever started at the age of 14 years. The fever was sudden in onset, remained around 40°C for 3 to 7 days and recurred monthly without strict periodicity. There were no evident triggers for the attacks.

The episodes were accompanied by fatigue, anorexia, abdominal pain, diarrhoea, and vomiting. He experienced arthralgias and later in the disease course, he had painful swellings of metacarpophalangeal and proximal interphalangeal joints. Rash, lymphadenopathy, hepatosplenomegaly had been absent. Serositis was suspected, since he suffered intestinal obstruction due to adhesions, without prior abdominal surgery. During attacks, he had leukocytosis with granulocytosis and band forms, and an elevated sedimentation rate and C-reactive protein. IgG, A, M, and D were elevated (table 2). Antibiotic treatment was ineffective and only long-term treatment with tolmetine and prednisolone could reduce, but not abort, symptoms.

At the age of 17 years, he died from the complications of a perforated gastric ulcer. At autopsy the gastric perforation and peritonitis were confirmed. An unexpected finding was a sterile pericarditis, further supporting the presence of polyserositis.

Case No 138: This 8-year Turkish boy had developed febrile reactions after childhood vaccinations but had otherwise been well during the first years of life. He suffered repeated bouts of fever from the age of 3½ until the age of 7 years. His fever recurred every 4 weeks. Onset was abrupt, but without chills. His fever was spiking and lasted 4 to 7 days, before resolving spontaneously. Accompanying symptoms were headache, anorexia, general malaise, abdominal pain and diarrhoea. Examination during attacks revealed cervical lymphadenopathy and hepatosplenomegaly, but no recurrent pharyngitis, uveitis, oral or genital ulcers. He had recurrent otitis media and streptococcal pharyngitis once, but most febrile episodes were without any recognisable infectious focus. There were no other affected family members.

Immunoglobulins were repeatedly elevated. Notably, IgD was persistently raised (231-415 IU/ml), despite clinical remission. Sequence analysis of the *MEFV*-gene was normal, rendering Familial Mediterranean Fever (FMF) unlikely and Behçet's syndrome was excluded on clinical grounds.

Case No 140: This 6 year-old Dutch boy had suffered repeated febrile attacks from the age of 3 months. Attacks were triggered by vaccinations and infections, but usually came unexpectedly, starting with chills and peripheral cyanosis followed by an sudden rise in temperature up to 41.4°C. Fever remained high during 3 to 5 days and recurred every 3 to 5 weeks. Over the last 12 months, his fever had not exceeded 38,5°C and attacks came 2 to 3 months apart. Fatigue and anorexia preceded the attacks. The crises were accompanied by abdominal pain, headache, vomiting, and cervical lymphadenopathy, but not by joint pains, rash, hepatosplenomegaly, oral ulcers, or pharyngitis. Once the fever had abated, the boy remained tired and listless for about 3 more days. Serum-IgG had ranged between 9.7 and 12.6 g/l, IgM between 0.8 and 2.2 g/l and IgA between 0.8 and 1.25 g/l. Serum-IgD was persistently elevated between 157 and 180 IU/ml. During attacks leukocytes, granulocytes, sedimentation rate and C-reactive protein were raised.

Apart from oral antibiotics, which seemed ineffective, no other medications had been tried.

In all 4 boys, the family history was negative for recurrent fever, making the autosomal dominant TNF-receptor associated periodic syndrome very unlikely. In three, the ethnic background did not suggest Familial Mediterranean Fever. Cyclic neutropenia was formally ruled out in all 4 and the PFAPA syndrome and systemic onset juvenile idiopathic arthritis were ruled out on clinical grounds. During most of the attacks, no infectious focus could be identified.

Discussion

Periodic fever in childhood is not uncommon. Currently, various clinical syndromes, characterised by periodic fever can be distinguished. These include the autosomal recessive syndromes HIDS and Familial Mediterranean Fever, the autosomal dominant TNF-Receptor Associated Periodic Syndrome (TRAPS), and the non-hereditary disorders Periodic Fever, Aphtous stomatitis, Pharyngitis, Adenitis (PFAPA) syndrome (9;10), and systemic onset juvenile idiopathic arthritis.

In the past these syndromes could only be diagnosed on the basis of clinical criteria. Recently, however, the molecular defects in HIDS (3;4), FMF (11;12), and TRAPS (13) have been identified. This knowledge now helps us to differentiate these disorders from each other and will provide better insight in the pathogenetic mechanisms involved in the various periodic fever syndromes.

The clinical presentation of most periodic fever syndromes shows considerable inter-individual variation. With the identification of the molecular defects, the question, whether this clinical heterogeneity reflects an underlying genetic heterogeneity, can be addressed. We investigated a group patients, originally diagnosed with HIDS by the occurrence of episodic fever in combination with an elevated serum IgD after the exclusion of other (infectious) causes for the fever. As we now know, HIDS is due to a deficiency of mevalonate kinase (MK), an enzyme involved in one of the early steps of isoprenoid biosynthesis. This biochemical pathway yields many substances, including cholesterol, dolichol, heme A, ubiquinone, and hydrophobic moieties for posttranslational protein modification (farnesyl- and geranyl-geranyl-groups). It is unknown how MK deficiency leads to recurrent febrile episodes. Our previous work has shown that MK deficiency can also give rise to periodic fever without raised IgD (3). Hence, in patients with unexplained periodic fever, MK activity should be determined irrespective of serum IgD.

To examine, whether our clinically diagnosed HIDS patients indeed had MK deficiency, we measured MK enzyme activity in all 15. MK was deficient in 11 patients. Urinary mevalonic acid excretion in HIDS is known to rise during fever, but it is not known whether MK activity can be adversely affected by influences such as fever or mental stress. Hence, enzyme activity measurements were performed only on blood, drawn between fever episodes. In the 11 MK deficient patients we found mutations in both alleles of *MVK*, the gene encoding MK. At the molecular level there was heterogeneity among the MK-deficient patients. All patients carried the V377I allele, which has previously been shown to lead to an unstable protein with reduced enzymatic activity (3). Two patients were homozygous for the V377I allele. The other patients were compound heterozygous for this allele and 1 of 4 other mutant alleles. Two of these, 59 A→C and 803T→C, are known not to produce an enzymatically functional protein (3). Patients homozygous for mutations that completely abolish MK activity are known to present with a distinct clinical syndrome, called mevalonic aciduria (MA). MA is characterised by mental retardation, facial dysmorphy, and failure to thrive, in addition to febrile crises (14). Apparently the V377I allele is responsible for the milder phenotype of HIDS as compared to classical mevalonic aciduria. The mutations 1162 C→T and 404 T→C had not been previously described in HIDS or MA. The predicted effect of the mutation 1162 C→T is a truncation of the protein at position 388. The functional effect of the S135L substitution, resulting from the mutation 404 T→C, is unknown. However, it is likely to lead to loss of function, since the serine residue at position 135 is highly conserved among mammalian species and the measured MK activity in the patient was only 1% of normal. A genotype phenotype relation exists within the broad spectrum of mevalonate kinase deficiency in that severe mutations leading to virtually abolished MK activity give rise to the MA phenotype, whereas mild changes lead to the HIDS phenotype. In contrast to findings in MA, none of the patients described here had less than 1% residual enzyme activity. Among

these, we could not establish a relation between the different mutations and residual MK activity. Neither could we relate the severity of clinical symptoms to the residual activity of MK. For example, 4 patients are compound heterozygous for I268T and V377I. Yet, the residual MK activity varies from 2% in patient 132 to 7% in patient 135. These 2 patients are both severely affected. On the other hand, patient no 137 is only mildly affected, although her residual MK activity is intermediate between that of patients No 132 and 135.

Interestingly, of the 15 children originally diagnosed with HIDS, 4 showed a normal MK activity, indicating that HIDS is a genetically heterogeneous disorder. These patients had been diagnosed as HIDS because of elevated IgD and recurrent fever. In 2 patients, however, the febrile episodes had been absent for more than 18 months. Although this is not uncommon in HIDS, it sets these cases apart from the MK-deficient patients in this report. Indeed, the symptoms of these 4 patients, all boys, differed slightly from our MK-deficient patients. The diagnosis of HIDS could not have been rejected on clinical grounds, however. The clinical phenotype reported in HIDS patients before MK testing became available (2) and in our MK-deficient patients is not uniform and the clinical spectrum of HIDS accommodates all the characteristics of these 4 boys.

In 2 patients with normal MK activity the onset of disease was beyond 3 years of age. In contrast, all MK deficient patients described here and 70.% of reported HIDS cases (2) had their onset of disease in the first year of life. Another difference was the absence of vomiting, arthralgias, and rash in 3 of 4 patients with normal MK activity, while these features were present in the majority of the MK deficient patients as well as in most reported HIDS patients. Therefore, although not obligatory in MK deficiency, the absence of absence of vomiting, arthralgias, and rash in a child with fever and elevated IgD seems to make MK deficiency less likely. The same applies to the absence of diarrhoea. Diarrhoea was present in all MK-deficient cases and only in 2 of 4 patients with normal MK activity. One patient without MK

deficiency was mentally retarded. However, 2 mentally retarded brothers with HIDS have been reported in the literature (2) who were later shown to be mevalonate kinase deficient (data not shown).

This study shows that HIDS is a heterogeneous disease. On the biochemical level most of the patients show a MK deficiency. Here we report on 4 patients, who were originally characterised as HIDS, but had a normal MK activity. Since the clinical differences were discrete, determination of MK activity is the only certain way to differentiate these patients from those who have MK deficiency. It remains to be determined, whether measurement of urinary mevalonic acid excretion is equally reliable. The distinction between these groups is important since MK deficiency is an autosomal recessive disease, with consequent implications for genetic counselling. The defect or defects in the patients with periodic fever, elevated IgD and normal MK activity are unknown at this moment. Despite the predominance of boys, an X-linked disorder is unlikely, in view of the negative family history. Due to the small size of Dutch families a negative family history does not rule out autosomal recessive disease, which is illustrated by the 11 MK deficient children, none of whom had affected siblings. One of the possibilities is that disorders of isoprenoid biosynthesis other than MK deficiency could also cause periodic fever. Investigations to test this possibility are underway.

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Table 1

Registry number [1]	26	61	72	88	119	129	132	135	137	139	142		*50*	*136*	*138*	*140*
Age (years)	18	8	6	9	8	11	8	12	2	5	5		*8*	*17*	*8*	*6*
Sex M/F	M	F	F	M	M	M	M	M	F	F	F		*M*	*M*	*M*	*M*
Disease onset (months)	3	1.5	3	5	3	3	6	0	11	3	12		*2*	*160*	*42*	*4*
Duration of fever (days)[2]	3	7	4	5	4	4	4	3	4	4	3		*3*	*4*	*7*	*4*
Duration of cycle (weeks)[3]	3	2	3	3	3	3	2	3	7	5	3		*3*	*4*	*4*	*8*
Malaise[4]	+	+	-	+	+	+	+	+	+	+	+		+	+	+	+
Chills	-	+	-	+	+	-	-	+	-	-	-		-	-	+	+
Fever on vaccinations	+	+	+	+	+	+	+	+	+	+	+		+	-	+	+
Headache	+	+	+	+	+	+	+	+	-	-	+		-	-	+	+
Abdominal pain	+	+	+	+	+	+	+	+	-	+	+		-	+	+	+
Vomiting	+	+	+	+	+	+	+	+	+	-	+		-	+	-	-
Diarrhoea	+	+	+	+	+	+	+	+	+	+	+		-	+	+	-
Arthralgias	+	+	+	+	+	+	+	+	+	+	+		-	+	-	-
Arthritis	-	-	-	-	-	-	+	+	-	+	-		-	+	-	-
Rash	+	+	+	+	+	-	+	+	+	+	+		-	-	-	+
Lymphadenopathy	+	+	+	+	+	-	+	+	+	+	+		+	-	+	+
Splenomegaly	-	+	-	-	+	+	+	+	-	+	+		-	-	+	-
Hepatomegaly	+	+	-	-	+	+	+	+	-	+	+		+	-	+	-

Table 2

Registry number	IgGmax[1] (g/l)	IgMmax[1] (g/l)	IgAmax[1] (g/l)	IgDmin[2] (IU/ml)[4]	IgDsubmax[3] (IU/ml)[4]	IgDmax (IU/ml)[3]	Urinary mevalonic acid[1,5]	Mevalonate kinase activity[6]	Mevalonate kinase allotype[7]
26	11.9	1.4	7.9	n.a.[8]	1680	2040	5.4	1%	V377I, H20P
61	24.5	3.1	2.3	210	571	1050	4.8	1%	V377I, S135L
72	8.1	0.5	1.6	585	805	826	n.a.[8]	7%	V377I, V377I
88	15.4	1.35	6.9	236	780	1107	22.4	4%	V377I, R388X
119	11.9	1.65	2.55	338	338	505	41.5	3%	V377I, H20P
129	17.3	2.8	1.9	550	571	1286	10.8	1%	V377I, H20P
132	18.6	2.42	2.38	3	370	450	12.1	2%	V377I, I268T
135	13.1	2.1	1.8	77	98	244	11.4	7%	V377I, I268T
137	15.4	3.3	0.85	76	81	179	27.5	5%	V377I, I268T
139	11.3	1.34	3.49	47	95	192	n.a[8]	5%	V377I, V377I
142	28.2	7.45	4.5	66	92	158	21.7	4%	V377I, I268T
50	*12.5*	*1.8*	*3.1*	*89*	*209*	*209*	*<2*	*112%*	*wild type*
136	*14*	*2*	*3.15*	*93*	*150*	*274*	*n.a.*[8]	*88%*	*wild type*
138	*13.6*	*1.8*	*2.73*	*231*	*394*	*415*	*<2*	*90%*	*wild type*
140	*12.6*	*2.2*	*1.25*	*157*	*160*	*181*	*<2*	*144%*	*wild type*

Table 3

Mutant allele	Coding effect	frequency
59 A→C	His 20→ Pro (H20P)	3/22
404T→C	Ser 135→Leu (S135L)	1/22
803T→C	Ile 268→Thr (I268T)	4/22
1129 G→A	Val 377→ Ile (V377I)	13/22
1162 C→T	Arg 338 →Stopcodon (R338X)	1/22

Table 1

Characteristics of the patients.

1. Number refers to the entry number in the international Hyper IgD studygroup registry.
2. Duration of fever: the usual average duration of attacks as reported by parents at the time of diagnosis.
3. Duration of cycle: the usual average duration between the onset of an attack and that of the next attack as reported by parents at the time of diagnosis.
4. A symptom is recorded as positive when it has been present with at least one attack. The data of patients with normal enzymatic activity are printed in italics.

Table 2

Immunoglobulin levels, mevalonic acid excretion, MK-activity and MK allotypes in 17 HIDS-patients.

1. Highest documented value
2. Lowest documented value
3. Second highest documented value
4. Normal < 100 IU/ml. Where IgD had been reported in mg/l, the value was divided by 1.4 to convert to IU/ml.
5. Urinary mevalonic acid excretion in mmol/mol creatinine
6. Expressed as percentage of average activity in mononuclear cells from healthy control persons, normal range (58-142%).
7. The allotypes were deducted from *MVK* cDNA mutation analysis (see text and table 3).
8. N.a.: not available

The data of patients with normal enzymatic activity are printed in italics.

Table 3

Mutations identified in the cDNA of 11 mevalonate kinase deficient patients and their effect on the mevalonate kinase protein . The frequency with which an allele was recognised among the 2x11 mutant alleles found is given in the last column.

Molecular basis of classical mevalonic aciduria and the hyperimmunoglobulinaemia D and periodic fever syndrome: High frequency of 3 mutations in the mevalonate kinase gene

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The classical type of mevalonic aciduria (MA; MIM# 251170) is a rare autosomal recessive metabolic disorder characterized by psychomotor retardation, failure to thrive, hepatosplenomegaly and anemia. In addition, patients suffer from recur rent episodes of fever, associated with lymphadenopathy, arthralgias, gastrointestinal problems and skin rash. The disorder is caused by deficient activity of mevalonate kinase (MK). As a consequence of the MK deficiency, high levels of mevalonic acid are present in plasma and urine of patients (1). Recently, mevalonic aciduria was also recognized in patients with hyperimmunoglobulinaemia D and periodic fever syndrome (HIDS; MIM# 260920), who also suffer from recurrent episodes of fever (2) (3). The mevalonic aciduria in HIDS patients, however, was moderate and observed only during the febrile crises. MK enzyme activity was decreased, but not fully deficient, varying between 1% and 4% residual activity (2). The

diagnostic hallmark of HIDS is a constitutively elevated level of serum IgD, usually accompanied by elevated levels of serum IgA (4). However, we recently identified patients with the classical HIDS phenotype and MK deficiency but normal serum IgD levels (2). Both syndromes are caused by mutations in the gene encoding MK. In HIDS, one missense mutation, 1129G >A, was common to most patients analyzed so far(2;5). Most patients were compound heterozygotes. At present, mutations in eight MA patients have been characterized (6-9). In three patients an 803T >C mutation was identified for which a Mennonite ancestry was suggested (7). This allele was also identified in two additional HIDS patients, suggesting a high frequency of this mutation.

We describe here the frequency of these two mutations and a third common mutation in the MK gene identified in eight new HIDS patients and calculate the allele frequency of these mutations in all reported HIDS and MA patients. In addition, we make a comparison between the genotypes of HIDS and MA patients.

MATERIALS AND METHODS

All patients studied were clinically diagnosed with HIDS and had strongly decreased activities of MK as measured in fibroblasts and/or lymphocytes making use of 14C-labelled mevalonate (10). Mutation analysis was performed on cDNA as described previously (9).

RESULTS AND DISCUSSION

Previous studies suggested that the 1129G >A mutation in the MK gene was the most common cause of HIDS. We sequenced eight additional HIDS patients and identified nine 1129G >A alleles. One patient was homozygous; this was confirmed by sequencing parental material. In addition, we identified four 803T >C alleles and one 59A >C allele. Both alleles had previously been reported in other HIDS and MA patients, indicating a high frequency of these alleles. Table 1 summarizes the frequency of these alleles in our patients and patients diagnosed in other laboratories. The table includes data of only one affected member per

family in those cases where more members have been identified. The 1129G >A mutation has an allele frequency of 52% among HIDS patients. At present, only one HIDS patient has been described who did not carry this allele (5). None of the MA patients carried the 1129G >A allele, strongly suggesting that this mutation is responsible for the residual MK activity measured in HIDS fibroblast lysates(2). Heterologous expression of this allele as a GST-MK fusion protein in *Escherichia coli* revealed reduced enzyme activity. In addition, immunoblot analysis showed a profound deficiency of the protein in lysates of skin fibroblasts of HIDS patients, suggesting instability of the product of this allele in vivo (2). The estimated frequency of the 803T >C allele among both HIDS and MA patients is 17%. Heterologous expression of this mutant allele in *E. coli* resulted in strongly reduced MK enzyme activity. Moreover, immunoblot analysis revealed low protein levels in an MA patient homozygous for this allele (9). The fact that this severely affected MA patient died at an early age suggests that the frequency of this allele might be underestimated owing to lethality in utero. This allele was also identified in the only HIDS patient without the 1129G >A mutation, which suggests that the second mutation identified in this patient, 500C >T, leads to the HIDS phenotype. Heterologous expression of the resulting mutant enzyme and protein level analysis by immunoblotting will be needed to determine the effect of this 500C >T mutation on the enzyme. The third common allele, 59A >C, which encodes an inactive protein when expressed in *E. coli*, has a frequency of 7% among HIDS and MA patients.

Table 1

Frequency of three common mutations in the MK gene in HIDS and MA patients

		Number of alleles in HIDS			Number of alleles in MA		
Coding Effect	*Mutation*	*Our laboratory (a) (24 alleles)*	*Other laboratories (b) (18 alleles)*	*Frequency (%)*	*Our laboratory (c) (6 alleles)*	*Other laboratories (d) (10 alleles)*	*Frequency (%)*
V377I	1129G>A	14	8	52	0	0	0
I268TI	803Y>C	5	1	14	2	2	25
H20P	59A>C	3	0	7	1	0	6

a Houten et al (2); this work

b Drenth et al (5)

c Houten et al (9)

d Schafer et al (8); Hinson et al (6;7)

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Lack of isoprenoid products raises ex-vivo interleukin-1β secretion in HyperImmunoglobulinemia D and periodic fever syndrome.

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running title: Lack of isoprenoids raises IL-1β in HIDS

ABSTRACT

Objective:

The Hyper IgD and periodic fever syndrome (HIDS) is an auto-inflammatory syndrome, in which ex-vivo secretion of pro-inflammatory cytokines, such as Interleukin (IL)-1β, by peripheral blood mononuclear cells (PBMC) is increased. HIDS is caused by a deficiency of mevalonate kinase (MK).

How this inborn error in isoprenoid biosynthesis leads to inflammation is as yet unknown.

We investigated whether the increased IL-1β secretion is due to accumulation of mevalonate, the substrate of the deficient enzyme or due to lack of its products, the isoprenoid compounds.

Methods:

The effects of lovastatin and of farnesol, geranylgeraniol, and mevalonate on PBMC from 8 patients with MK deficiency and from 13 controls were studied. Lovastatin inhibits isoprenoid biosynthesis by reducing the production of mevalonate. Farnesol and geranylgeraniol restore isoprenoid biosynthesis downstream from MK. Culture supernatants were collected for cytokine analysis 48 hours after stimulation with monoclonal antibodies against CD2 + CD28.

Results:

Lovastatin induced a 9-fold rise in IL-1β secretion by normal anti-CD2 + CD28 stimulated cells ($p < 0.001$). This effect could be countered by mevalonate, and, to a lesser extent, by farnesol and geranylgeraniol. In the absence of lovastatin, mevalonate did not change IL-1β secretion.

MK deficient cells spontaneously secreted 9-fold more IL-1β than control PBMC ($p < 0.005$), rising 2.4-fold in the presence of lovastatin. The effect of lovastatin on IL-1β secretion was reduced by mevalonate, farnesol and geranylgeraniol.

Isoprenoid biosynthesis in PBMC from patients is impaired due to the endogenous MK deficiency. Bypassing this defect with farnesol, in the absence of lovastatin, led to a 62% reduction ($p < 0.02$) in IL-1β secretion by these cells.

Conclusions

In this model, shortage of isoprenoid end products contributes to increased IL-1β secretion by MK deficient PBMC, whereas excess mevalonate does not.

Key words

Mevalonic acid, fever, hyperimmunoglobulinaemia, immunoglobulin D, autoinflammatory, Interleukin-1, cytokines, inflammation, lovastatin, farnesol.

INTRODUCTION

The hyperimmunoglobulinemia D and periodic fever syndrome (HIDS), also known as Dutch type periodic fever (MIM#260920), is an autosomal recessive disorder, characterized by febrile attacks recurring at more or less regular intervals and the presence of an elevated serum IgD concentration (> 100 IU/ml)[1]. Over 170 patients have been diagnosed with the disease worldwide [2]. Clinical features during the febrile attacks include cervical lymphadenopathy, splenomegaly, hepatomegaly, skin rash, oral ulcers, vomiting, diarrhea, arthralgias, and arthritis. Patients often complain of malaise, chills, headache, nausea or abdominal pain [1]. During these febrile crises, blood tests reflect an acute inflammatory state, with leukocytosis and elevated acute phase reactants e.g. C reactive protein.

Serum levels of pro-inflammatory cytokines, such as IL-6, and interferon gamma (IFN-γ), rise during fever attacks [3;4]. Also, between attacks, isolated peripheral blood mononuclear cells (PBMC) from HIDS patients secrete large amounts of pro-inflammatory cytokines, such as interleukin (IL)-1β[5]. However, what causes the rise in production of these cytokines has remained unclear.

In 1999, we and others identified the underlying genetic defect of the syndrome, namely mutations in the gene *MVK*, which lead to a deficiency of the enzyme mevalonate kinase[6;7] (MK). Mutations in the same gene are responsible for mevalonic aciduria (MA, MIM#251170), a syndrome with episodic fever, mental retardation and dysmorphic features[8;9]. MK catalyses the phosphorylation of mevalonic acid into 5-phosphomevalonate. It is the enzyme immediately following 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase in the isoprenoid biosynthesis pathway (Fig. 1)[10].

This pathway produces cholesterol, which, in turn, is a precursor for steroid hormones, and bile acids. Furthermore, this biosynthetic route yields a number of non-sterol isoprenoids. The latter are hydrophobic molecules, such as dolichol and the polyisoprene side chains of heme-A and ubiquinone. Non-sterol isoprenoids play a vital role in the prenylation of certain proteins. In this process hydrophobic polyisoprene moieties are covalently attached to specific target proteins. Prenylation comes in two forms, the attachment of farnesyl groups (from farnesylpyrophosphate) or of geranylgeranyl groups (from geranylgeranylpyrophosphate).

In HIDS, the deficiency of MK causes accumulation in plasma of its substrate, mevalonic acid, which is then excreted in the urine [6]. The causal link between mevalonate kinase deficiency and inflammation remains to be clarified. Inflammation has to result from either the excess of mevalonic acid or the shortage of one or more products of isoprenoid biosynthesis.

Recently, it has become apparent that impairment of isoprenoid biosynthesis can indeed influence immune function. In vitro, inhibitors of HMG-CoA reductase, also known as statins, may either suppress or enhance inflammatory responses, depending on the cell type studied and the way in which these cells were stimulated. Many anti-inflammatory effects of statins have been reported, including the reduction of lymphocyte proliferation, and of the expression of MHC class II molecules, matrix metalloproteinases, cytokines, and chemokines [11-15]. On the other hand, these compounds do have pro-inflammatory properties. Statins enhance endothelial expression of cellular adhesion molecules [16]. Interestingly, the secretion of IL-1β, Interferon-γ (IFN-γ) and IL-18 by PBMC stimulated ex-vivo with inactivated *M. tuberculosis* is augmented greatly by the inhibition of isoprenoid biosynthesis with statins[17]. The increased cytokine secretion appeared to be T-helper1-cell-induced and to be due to lack of isoprenoids, since addition of mevalonic acid reduced cytokine secretion to control levels.

The question is, whether the reported increased secretion of pro-inflammatory cytokines in HIDS could be explained along similar lines. If lack of isoprenoids were responsible, then addition of isoprenoid intermediary metabolites to cells from HIDS patients should correct the cytokine hypersecretion.

To be effective, these compounds should enter the isoprenoid pathway downstream from the metabolic block in HIDS, i.e. beyond mevalonate kinase. Farnesyl pyrophoshate (FPP) and geranylgeranyl pyrophosphate (GGPP) are such intermediary metabolites. Entry in living cells is impaired, however, by the presence of hydrophilic pyrophosphate groups. The fatty alcohols Farnesol (FOH) and geranylgeraniol (GGOH) are hydrophobic molecules that enter living cells freely and, once inside, are converted into FPP and GGPP by two successive monophosphorylations[18]. Therefore we studied the effects of FOH, GGOH, and mevalonate in addition to lovastatin on the IL-1β secretion by

PATIENTS AND METHODS

Patients

Pediatric HIDS or MA patients visiting our outpatient clinic for regular follow-up were approached to participate in the study. After approval by the ethical review board and written informed consent by the patients parents, blood was drawn by venipuncture in sterile pyrogen-free heparinized plastic tubes. All patients were free of symptoms and not taking anti-inflammatory drugs at the time of blood sampling. Healthy adult volunteers served as controls. PBMC were isolated by Ficoll (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. We incubated 1 x 10^5 cells per well in 96-well-flat-bottom microtiter plates in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 5 % heat inactivated fetal calf serum (Life Technologies, Grand Island, NY) filtrated through a 0.2 μm filter, 2 mM L-glutamine and 1% penicillin-streptomycin. Incubations were performed in quadruplicate at 37 °C in a humidified atmosphere containing 5 % CO_2 in air. The cultures were performed in the presence or absence of lovastatin (final concentration: 5 μM). In addition, either 1 mM mevalonate, 10 μM farnesol (FOH), 10 μM geranylgeraniol) GGOH, or 5 μM FOH/ 5 μM GGOH or medium was added at the start of the experiments. Lovastatin, mevalonic acid, FOH and GGOH were purchased from Sigma (St. Louis, MO). Both lovastatin, dissolved in ethanol, and mevalonic acid lactone were converted to lovastatin and mevalonate respectively by hydrolyzation (0.1M NaOH), followed by neutralization and stabilization at pH7.4 with 0.05M HEPES and HCl (0.1M).

After 18 hours incubation either *Mycobacterium tuberculosis,* Purified Protein Derivative (PPD) of *M. tuberculosis* (Statens Serum Institut, Copenhagen, Denmark), mycobacterial heat shock protein HSP60 (Sanbio, San Diego, CA), anti-CD2 + CD28 (PeliCluster CD 2.1, CD 2.2 and CD

28, CLB Amsterdam, The Netherlands) or medium was added to the cultures without any other change in the medium. The incubations were prolonged for an additional 48 hours. At the end of the incubations supernatants were aspirated and stored at -20 °C until analysis.

Cytokine measurements

Cytokine concentrations were measured in thawed samples, using commercially available enzyme-linked immunosorbent assays (PeliKine-compact^{TM} human IFN-γ, IL-1β, IL-6 and TNF-α ELISA kits CLB Amsterdam, The Netherlands). The assays were performed according to the manufacturer's instructions and all samples were tested in duplicate

Lymphocyte proliferation

PBMC at a concentration of 1 x 10^5 cells/well were cultured in 96 well flat-bottom microtiter plates using the conditions as described above. After 48 hours of incubation, cells were pulse-labeled with 1 μCi [^{3}H] thymidine for 18 hours at 37 °C. Cells were harvested and [^{3}H] thymidine incorporation was measured in a liquid scintillation counter. Results were shown as average counts per minute (cpm) of quadruplicate cultures.

Statistical analysis

The cytokine production under different conditions was compared by the Wilcoxon squared rank test, with a 2-tailed p value of < 0.05 being considered significant. Lymphocyte proliferation was analyzed similarly. Values are displayed as mean +/- the standard deviation. All analyses were performed using GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego, CA)

RESULTS

Patients

Seven HIDS patients participated in the study. These have been described previously, as Numbers 119, 132, 135, 137, 139, 142 and 192 [19].

In all patients mevalonate kinase deficiency has been established and mutations have been identified in both alleles of their MVK-genes. Two patients (a brother and sister) were homozygous for the 1129 G > A (V377I) mutation, four patients were compound heterozygous for the mutant alleles V377I and 803 T > C (I268T), and one patient carried the V377I and 59 A > C (H20P) alleles. The mevalonic aciduria patient was a 2 year old boy, who presented with episodes of high grade fever, anorexia, vomiting and macular rash. His mental development had been adequate. His head circumference, though still normal, has grown slowly, but weight and height gain have been excellent. He has no dysmorphic features. During febrile attacks he shows markedly elevated acute phase reactants and neutrophil leukocytosis. Urine mevalonic acid was extremely elevated (5461μmol/mmol creatinine). Mevalonate kinase activity in fibroblasts was 0.18 pmol/min.mg (0.12% of normal). He is compound heterozygote for the mutant alleles 1000G > A (A334T) and 421-422insG.

Cytokine secretion induced in normal mononuclear cells by anti-CD2 + CD28 can be augmented by lovastatin.

We tested the ability of *M. tuberculosis* and several T-cell stimuli to induce statin sensitive cytokine secretion (Fig. 2a). In the absence of lovastatin, the secretion of IL-1β was not significantly stimulated by *M. tuberculosi* , PPD, anti-CD2 + CD28, or HSP60. Lovastatin

alone, i.e. without added T-cell stimuli, hardly induced any increase in IL-1β secretion (Fig. 2b). However in the presence of both lovastatin and T-cell stimuli, IL-1β secretion rose 7-fold (PPD) to 15-fold (anti-CD2 + CD28).

IFN-γ secretion was stimulated by *M. tuberculosis*, PPD, and by anti-CD2 + CD28.The latter stimulus (anti-CD2 + CD28) elicited the strongest response. Combination with lovastatin induced a modest further increase of IFN-γ secretion, but this did not reach significance (data not shown). These stimuli did not induce secretion of IL-6. Neither did the secretion of IL-6 change upon incubation with lovastatin (data not shown).

Since the anti-CD2 + CD28-induced cytokine secretion was equal or superior to that induced by *M. tuberculosis*, PPD, or HSP60 and since this secretion was augmented consistently by lovastatin, only anti-CD2 + CD28 was used as stimulus in all subsequent experiments

Cytokine secretion is not augmented by addition of mevalonate per se

In order to evaluate whether the augmented IL-1 secretion was due to accumulated mevalonate in HIDS cells, the effects of exogenous mevalonate was studied in normal cells. In pilot experiments we had determined that 1 mM mevalonate was sufficient to overcome the antiproliferative effect of lovastatin without being toxic to the cells. In all subsequent experiments, the 1 mM concentration of mevalonate was used.

In the absence of lovastatin, mevalonate induced no detectable effect on the secretion of IL-1β (Fig. 2b), IFN-γ or IL-6. This implies that an excess of mevalonic acid is not responsible for the increased cytokine secretion observed in HIDS.

Mononuclear cells from HIDS and MA patients produce more IL-1β than control cells

Control cells excrete only small amounts of IL-1β (165 +/- 169 pmol/ml) when stimulated with anti-CD2 + CD28. PBMC from HIDS patients excrete larger amounts of IL-1β (1685 +/- 1713 pmol/ml) than controls ($p < 0.04$). Cells from the mevalonic aciduria patient produced even more IL-1β (4563 pmol/ml). HMG CoA reductase inhibition by lovastatin causes a further elevation of IL-1β excretion by anti-CD2 + CD28-activated cells from both patients (2.4-fold rise; $p < 0.05$) and controls (9.4-fold rise; $p < 0.01$) (Fig. 3). The spontaneous secretion of IL-6, known to be elevated in HIDS, was also raised in MA (17028 pg/ml vs 2260 pg/ml in controls). IFN-γ secretion in patients and controls was only detectable after anti-CD2 + CD28 stimulation and tended to be higher (though not significantly) in HIDS cells than in control cells. IFN-γ secretion by MA cells exceeded that of normal controls 3-4 fold.

The effect of lovastatin can be reversed by isoprenoid compounds, downstream from mevalonic acid.

From previous experiments [17] it had become clear that mevalonic acid could abort the increase in cytokine secretion, mediated by lovastatin. To see whether this was an effect of mevalonic acid per se or one of its downstream metabolites, we studied the ability of FOH and GGOH to correct this increased secretion.

In the supernatants of anti-CD2 + CD28 stimulated PBMC from normal healthy volunteers, IL-1β secretion was increased 9-fold when cultured in the presence of lovastatin (Fig. 4a). Mevalonate almost completely reversed this effect, reducing IL-1β secretion in the presence of lovastatin by 88% ($p < 0.01$). FOH, also reduced IL-1β secretion by 45%. ($p < 0.01$). The effect of GGOH was not statistically significant.

These results indicate that compounds downstream of mevalonate in the isoprenoid biosynthetic route can, at least in part, reverse the effect of lovastatin on IL-1β secretion. This implies that the lack of such compounds might be responsible for the increased cytokine secretion observed in the presence of lovastatin

IL-1β secretion in HIDS and MA is reduced by isoprenoid compounds downstream from mevalonic acid

In the presence of lovastatin, IL-1β secretion by PBMC from HIDS patients is reduced by the addition of FOH, GGOH or mevalonate (Fig. 4b). This effect is comparable to that observed in normal controls. The single patient with MA displayed a similar pattern (Fig. 4c).

Apparently decreasing mevalonate availability by the addition of HMG-CoA reductase inhibitors reduces isoprenoid output in HIDS and MA. This implies that, although affected patients are ill due to the reduced mevalonate kinase activity, there is an important residual MK activity and the enzyme does not become entirely and solely rate limiting.

The central question therefore was, whether downstream isoprenoids could correct the inherently raised secretion of IL-1β in HIDS and MA. Indeed, in the absence of lovastatin, i.e. with the endogenous mevalonate kinase deficiency being the only restriction on isoprenoid biosynthesis, downstream isoprenoids could reduce IL1-β secretion (Fig. 5). In this situation, mevalonate was, as expected, least effective, but FOH significantly reduced IL1-β secretion by 62% ($p < 0.02$).

anti-CD2 + CD28 induced T-lymphocyte proliferation is impaired by lovastatin

The rise in IL-1β secretion in culture supernatants could not be explained by higher cell numbers per culture well. Indeed, cell proliferation dropped by 62% in the presence of lovastatin ($p < 0.001$) (Fig. 6). Mevalonic acid completely reversed this effect. GGOH partially corrected the effect of lovastatin. This effect was modest (15%), but significant ($p < 0.01$).

These data indicate that in this system lovastatin is effective and that its effect can be antagonized by downstream isoprenoids in the concentrations employed.

DISCUSSION

. In recent years the genes involved in 4 hereditary periodic fever syndromes have been identified[6;7;20-23]. For two of these, *MEFV*, the gene mutated in familial Mediterranean fever and *CIAS1*, the gene affected in both familial cold-induced autoinflammatory syndrome and the Muckle-Wells syndrome, the functions are still unknown. In the TNF receptor associated periodic syndrome, the link with inflammation is straightforward, since the affected gene encodes the 55kDa receptor for tumor necrosis factor-α. In mevalonic aciduria and HIDS, the picture is more complex.

HIDS is a well defined autoinflammatory disease[24]. There are recurrent episodes of inflammation, reflected both in clinical signs and symptoms and in neutrophil leukocytosis and raised acute phase reactants [1]. Between attacks, serum IgD and IgA are usually raised and PBMC from HIDS patients secrete increased amounts of pro-inflammatory cytokines, notably TNF-α and IL-1β. The gene affected in both conditions, *MVK*, encodes mevalonate kinase, an enzyme involved in isoprenoid biosynthesis. However, how mevalonate kinase deficiency causes inflammation is not known at present. The enzyme deficiency leads to an increase of its substrate, mevalonate, and may lead to a shortage of end products, the isoprenoids (Fig. 1).

We adapted a model developed by Montero et al. [17] to test whether the increased cytokine secretion in HIDS could be explained by the elevated mevalonate levels and / or decreased isoprenoid production. In this model, isoprenoid output is reduced by lovastatin, which inhibits HMG-CoA reductase, the enzyme preceding mevalonate kinase in the isoprenoid biosynthetic pathway.

PBMC, stimulated with *M. tuberculosis* in the presence of lovastatin reportedly secreted more IFN-γ, IL-1β and IL-18 [17].

Using a combination of T-cell stimulating monoclonal antibodies against CD2 and CD28, we found that lovastatin causes a nine fold increase in IL-1β secretion by normal PBMC, though a similar effect on IFN-γ secretion did not reach significance. Mevalonate, by itself, did not induce IL1-β secretion. These observations therefore argue against mevalonate intoxication as the cause of increased IL-1β in HIDS.

To test whether downstream isoprenoid metabolites could correct this hypersecretion of IL-1β, we added either mevalonate, a precursor of farnesylpyrophosphate (FOH), or a precursor of geranylgeranylpyrophosphate (GGOH) to the culture medium. Mevalonate corrects all branches of isoprenoid synthesis, FOH, corrects mainly protein farnesylation and, to a lesser extent, synthesis of other sterol and non-sterol isoprenoids, GGOH predominantly corrects geranylgeranylation of proteins.

Spontaneous IL-1β secretion was elevated in PBMC from patients with HIDS and the one patient with MA. The combination of lovastatin and anti-CD2 + CD28 induced a rise in IL-1β output in both patients and controls. Mevalonate prevented this rise. Downstream isoprenoid metabolites largely compensated the effect of lovastatin. This implies that some isoprenoid product or products are necessary to prevent IL-1β secretion in this system. Which isoprenoid end products are ultimately involved and how these interact with inflammatory pathways is the subject of current research.

The finding that in HIDS cells that are largely mevalonate kinase deficient, inhibition of HMG-CoA reductase would be of any consequence and that mevalonate could correct the effect of lovastatin, is intriguing. Apparently, the residual MK activity (2 - 8%) is sufficient for changes in the availability of mevalonate to be reflected in isoprenoid biosynthesis. Even in MA, the enzyme deficiency, though profound, is not absolute and can, to a certain degree, be overcome

by raised mevalonate levels. The main question was, however, what this means for the situation in HIDS, i.e. in the absence of lovastatin. There, the endogenous mevalonate kinase deficiency is the only impairment to isoprenoid biosynthesis. We showed that, in the absence of lovastatin, FOH could inhibit the IL-1β production by HIDS cells.

We could not, however, reduce the spontaneous IL-1β secretion in HIDS to normal levels. This could be due to insufficient rescue of isoprenoid biosynthesis in this model. In that case higher concentrations of isoprenoids than those employed in the present study might be more effective. Alternatively, mechanisms less directly related to reduced isoprenoid output, may contribute to IL-1β hypersecretion in HIDS.

There is presently no effective treatment for either HIDS [2] or MA. If excess of mevalonic acid were the cause of symptoms, then treatment with statins could be beneficial. A trial to that end is underway in The Netherlands [2]. Experience with statins in MA has been disappointing, however, with severe inflammatory attacks apparently provoked by the drug (9). Our findings provide an explanation for this effect, since it is indeed the lack of downstream isoprenoids that contributes to increased IL1β secretion in HIDS.

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Legends to figures

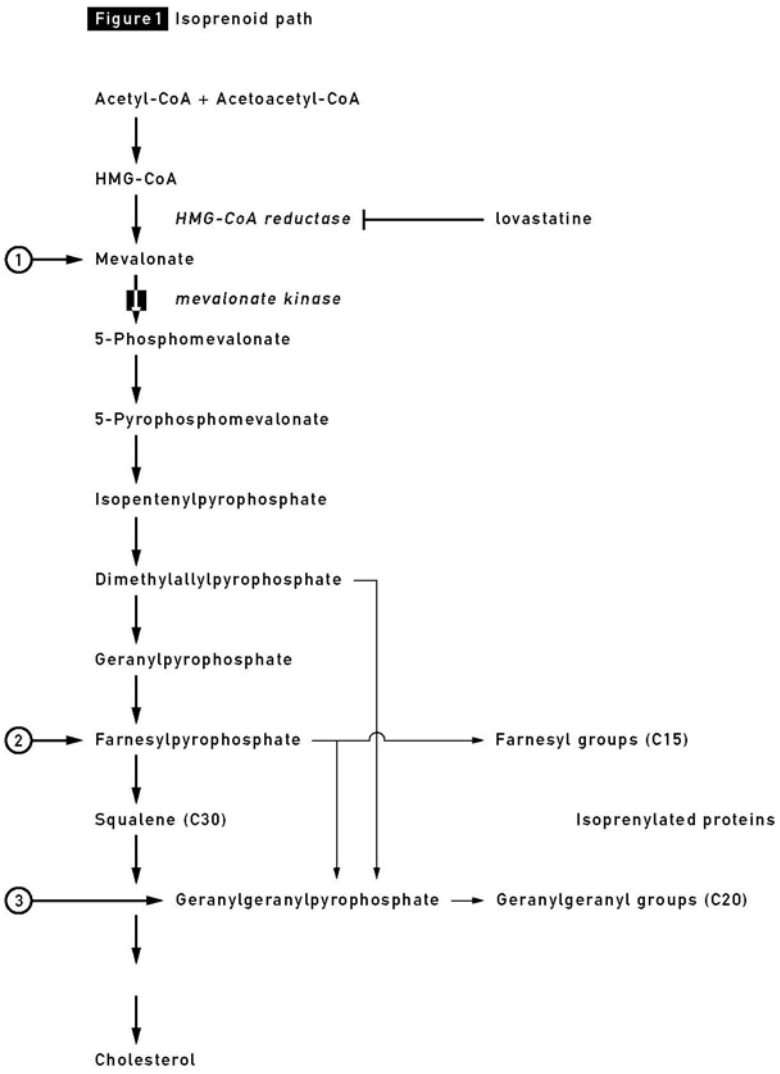
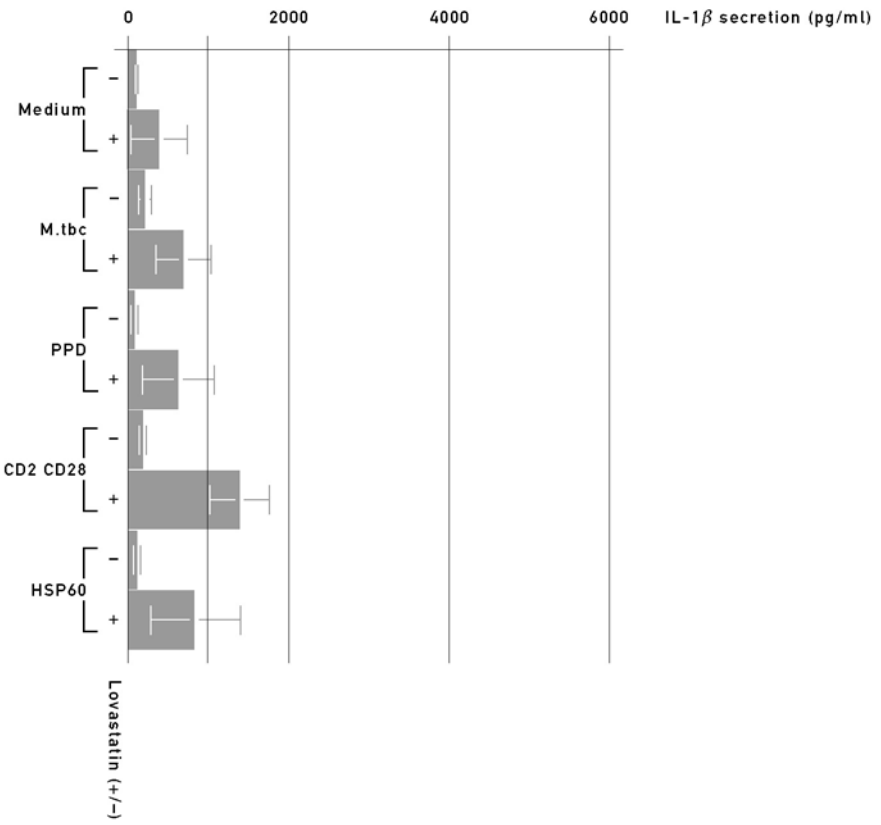
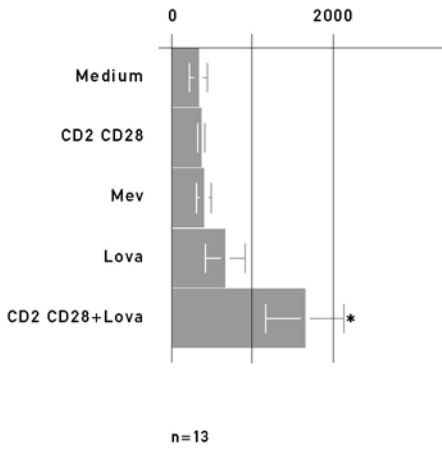


Figure 1

Isoprenoid biosynthesis pathway. Statins inhibit the early enzyme, HMG-CoA reductase. Numbers represent: 1: the defect in HIDS and MA is a deficiency of mevalonate kinase, 2: point of entry of farnesol (FOH), a precursor of farnesyl pyrophosphate and 3: point of entry of geranylgeraniol (GGOH) a precursor of geranylgeranyl pyrophosphate.

Figure 2

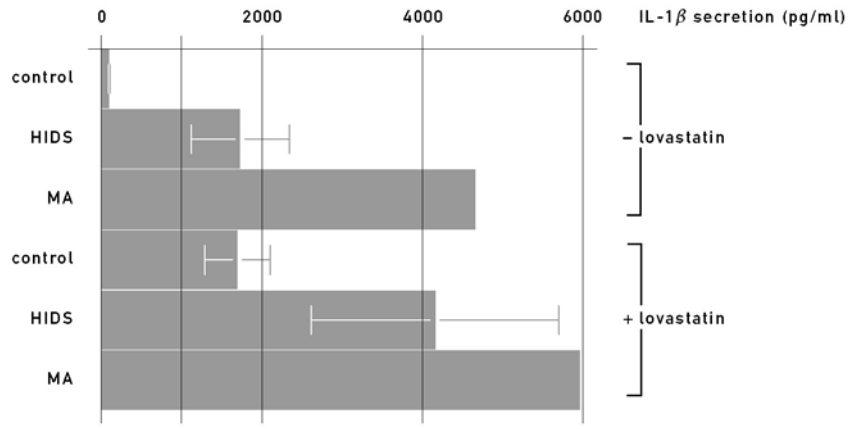




a) Mean interleukin (IL)-1β secretion by mononuclear cells from healthy adult controls (n=2) incubated in the absence (-) or presence (+) of 5 μM lovastatin. T-cell stimuli were none (Medium), *M. tuberculosis* (M.tbc), PPD of *M. tuberculosis* (PPD), anti-CD2 + CD28 (CD2CD28), and mycobacterial heat shock protein (HSP 60).Error bars represent standard deviation (SD)

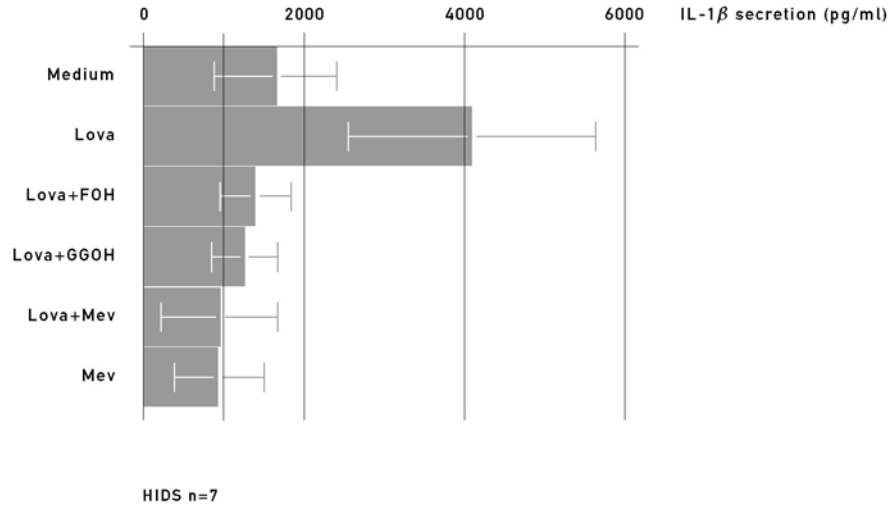
b) IL-1β secretion by normal mononuclear cells incubated with (from left to right) medium control, anti-CD2 + CD28, 1mM mevalonate, 5 μM lovastatin, or both lovastatin and anti-CD2 + CD28. Data are means + 1SD from 13 donors tested in 6 independent experiments. Values significantly ($p < 0.05$) different from the IL-1β concentrations obtained with medium only are marked (*).

Figure 3

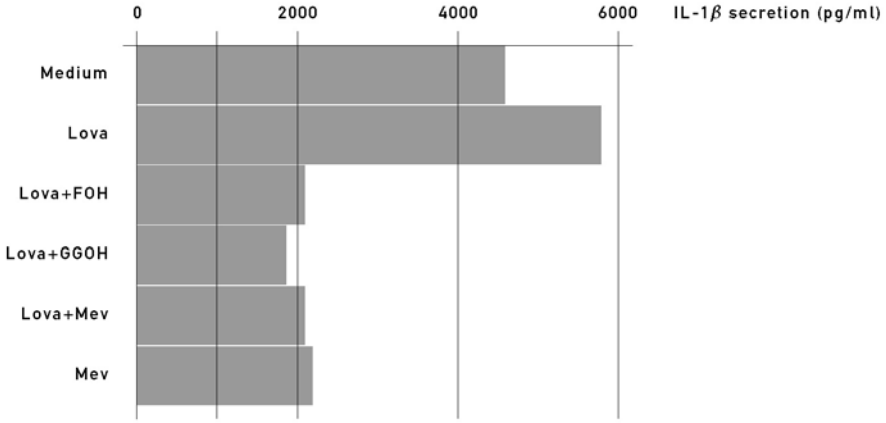


IL-1β secretion (mean + 1SD) by mononuclear cells from 13 controls, 7 hyper IgD syndrome (HIDS) patients and one mevalonic aciduria (MA) patient. Cells were incubated for 18 hrs in the absence (left panel) or presence (right panel) of 5 μM lovastatin and stimulated for 48 hrs with anti-CD2 + CD28.

Figure 4


0
2000
4000
6000
IL-1β secretion (pg/ml)
Medium
Lova
Lova+FOH
Lova+GGOH
Lova+Mev
Mev

HIDS n=7


0
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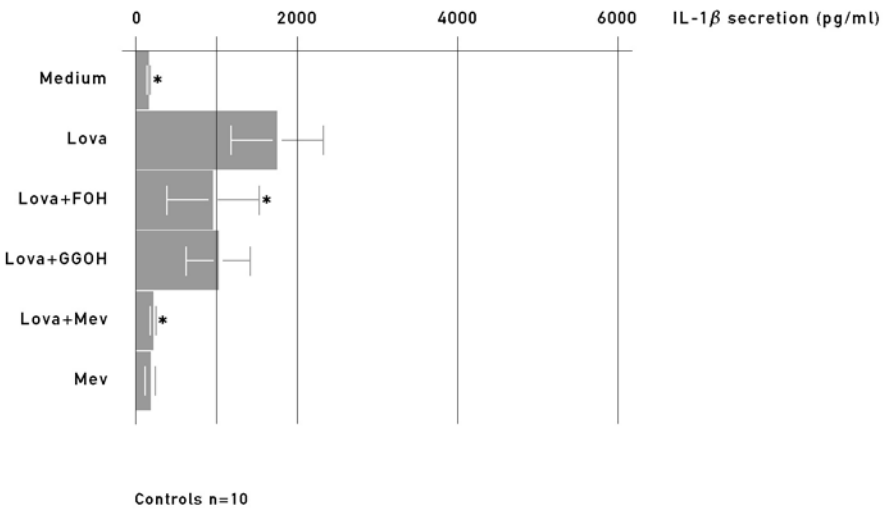
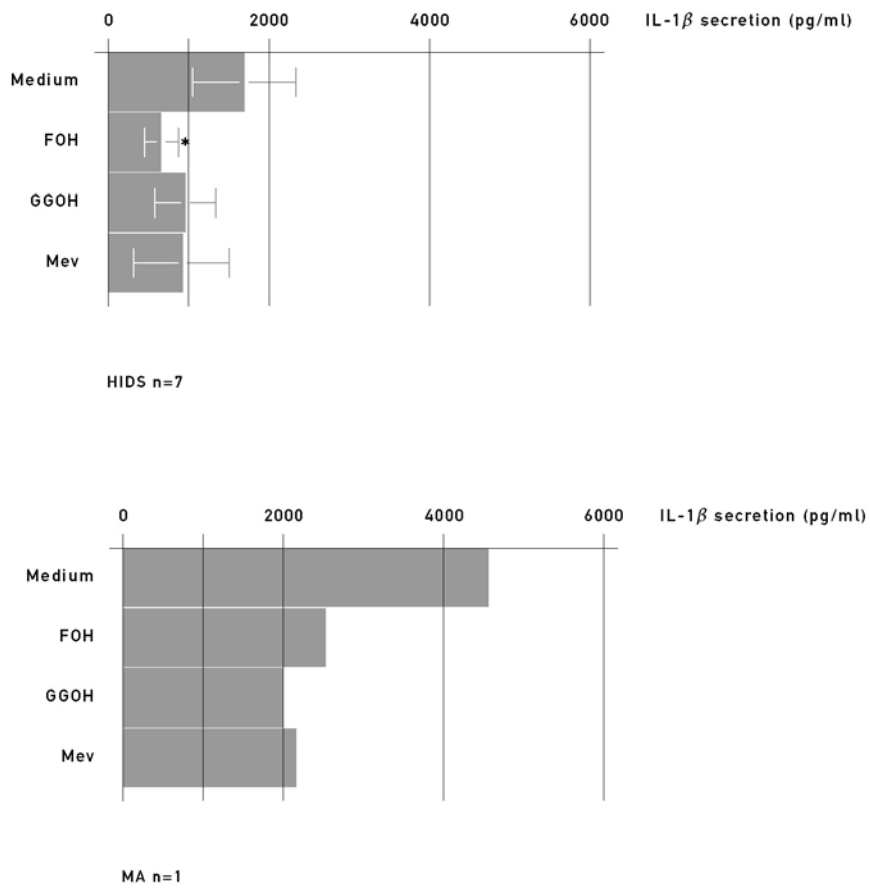
MA n=1



IL-1β secretion (mean + 1SD) by mononuclear cells, stimulated with anti-CD2 + CD28, from adult controls (panel a, n = 10), HIDS patients (panel b, n = 7) and one MA patient (panel c). Cells were incubated in the presence of culture medium only (Medium) or with the indicated combinations of 5 μM lovastatin (Lova), 10 μM Farnesol (FOH), 10 μM Geranylgeraniol (GGOH), 1 mM mevalonate (Mev).

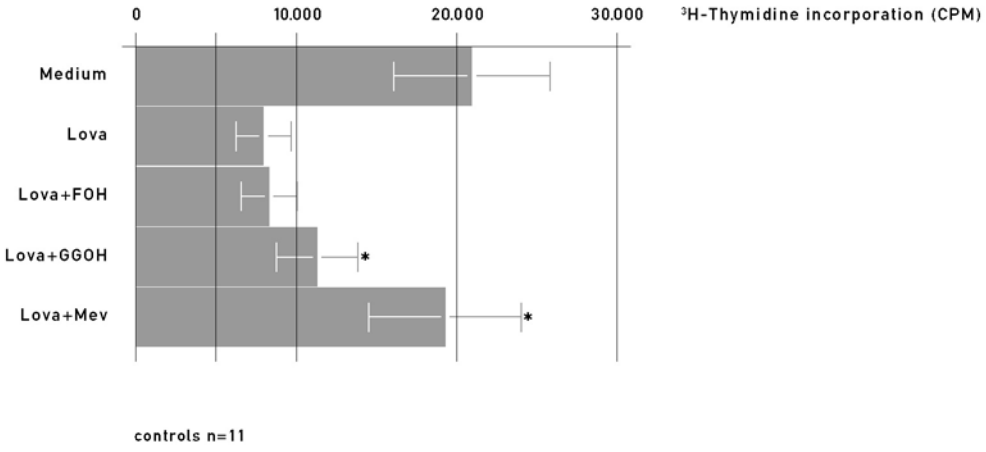
Values significantly ($p < 0.05$) different from the IL-1β concentrations obtained with the combination of lovastatin and anti-CD2 + CD28 are marked (*).

Figure 5



IL-1β secretion (mean 1SD) by mononuclear cells from HIDS patients (panel a, n = 7) and one MA patient (panel b), stimulated with anti-CD2 + CD28.in the absence of lovastatin. Cells were incubated in the presence of culture medium only (Medium), 10 μM FOH, 10 μM GGOH, or 1 mM mevalonate (Mev).Values significantly ($p < 0.05$) different from the IL-1β concentrations obtained with only anti-CD2 + CD28 stimulation are marked (*).

Figure 6



Proliferation of mononuclear cells from controls (n = 11), stimulated with anti-CD2 + CD28.

Bars represent mean ^{3}H-thymidine incorporation + 1SD Cells were incubated in the presence of culture medium only (Medium), or in the presence of the indicated combinations of 5 μM lovastatin (Lova), 10 μM FOH, 10 μM GGOH, 1 mM mevalonate (Mev).

Values significantly ($p < 0.05$) different from the ^{3}H-thymidine incorporation obtained with the combination of lovastatin and anti-CD2 + CD28 are marked (*).

Leukocyte activation in the HyperImmunoglobulinemia D and periodic fever syndrome.

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running title: Leukocyte activation in HIDS

Abstract

Objective:

The Hyper IgD and periodic fever syndrome and mevalonic aciduria are, characterized by recurrent episodes of generalized inflammation. Both syndromes are caused by a deficiency of mevalonate kinase. How this inborn error in isoprenoid biosynthesis leads to inflammation and which cells are involved in this process is as yet unknown.

We investigated whether specific leukocyte populations are activated during the fever attacks in children with mevalonate kinase deficiency.

Methods:

Blood samples obtained during and in-between fever attacks were analyzed by white-cell and differential counting and by flow-cytometry. Cells were studied for the expression of CD3, CD4, CD8, CD14, CD20, CD23, CD64, CD69 and HLA-DR.

Results:

6 patients were studied. During fever monocyte numbers rose 3-fold and neutrophil granulocytes 4-fold. These cells were activated, as reflected by the expression of CD64, which was increased 3-fold on monocytes and 6-fold on granulocytes. There were no such changes in other leukocyte subsets.

Conclusions

Activation of monocytes and neutrophil granulocytes is involved in the fever attacks of the Hyper-IgD and periodic fever syndrome.

Key words

Mevalonic acid; fever; hyperimmunoglobulinaemia, immunoglobulin D; autoinflammatory; inflammation; leukocytes; surface antigens.

Introduction

The hyperimmunoglobulinemia D and periodic fever syndrome (HIDS), also known as Dutch type periodic fever (MIM#260920), is an autosomal recessive disorder, characterized by febrile attacks recurring at more or less regular intervals and the presence of an elevated serum IgD concentration (>100 IU/ml)(1). Over 170 patients have been diagnosed with the disease worldwide (2). Clinical features during the febrile attacks include cervical lymphadenopathy, splenomegaly, hepatomegaly, skin rash, oral ulcers, vomiting, diarrhea, arthralgias, and arthritis. Patients often complain of malaise, chills, headache, nausea or abdominal pain (1). During these febrile crises, blood tests reflect an acute inflammatory state, with leukocytosis and elevated acute phase reactants e.g. C reactive protein. The underlying genetic defect of the syndrome, is a deficiency of the enzyme mevalonate kinase (MK) due to mutations in its encoding gene, *MVK*.(3,4). Mutations in the same gene are responsible for mevalonic aciduria (MA, MIM#251170), a syndrome with episodic fever, mental retardation and dysmorphic features(5,6). MK catalyses the phosphorylation of mevalonic acid into 5-phosphomevalonate, an early step in the isoprenoid biosynthesis pathway. This route produces cholesterol, which, in turn, is a precursor for steroid hormones, bile acids and vitamin D. Furthermore, this anabolic route yields a number of non-sterol isoprenoids. The latter are hydrophobic molecules, such as dolichol, the polyisoprene side chains such as those attached to heme-A and ubiquinone and the farnesyl and geranylgeranyl side chains, of isoprenylated proteins. Presently, the chain of

events that links this metabolic defect to episodic inflammation is understood only partly. Indeed, much has been learned about the soluble pro- and anti-inflammatory mediators, involved in HIDS. Patient serum contains high levels of pro-inflammatory cytokines, such as interferon-γ and interleukin (IL)-6 (7,8) during fever attacks and ex-vivo, isolated mononuclear cells (MNC) from HIDS patients secrete more IL-1β, IL-6, and TNF-α than MNC from healthy individuals.

It is as yet not known which leukocyte subpopulation(s) are involved in the inflammatory activation that characterizes HIDS. We therefore analyzed leukocyte (sub)populations in peripheral blood of patients during and in-between fever attacks.

Our aim was to establish, whether there were quantitative changes in leukocyte subpopulations and whether one or more of these cell types appeared to be activated in mevalonate kinase deficiency.

Patients and methods

Pediatric mevalonate kinase deficiency patients with either the HIDS or the MA phenotype, visiting our outpatient clinic for regular follow-up, were approached to participate in the study. After approval by the ethical review board and written informed consent by the patients parents, blood was drawn by venipuncture in sterile pyrogen-free heparinized plastic tubes as well as in 500 µl EDTA- anticoagulated plastic cups . This was done both when patients were free of symptoms and within the first 24 hours of a febrile attack. EDTA samples were used for white blood cell counting and white cell differential counting (Cell-Dyn 4000, Abbott Diagnostics, Santa Clara, CA). The heparinized samples were kept on ice from the moment of venipuncture until flowcytometric analysis, in order to prevent granulocytes from being activated. Patients were not taking anti-inflammatory drugs at the time of blood sampling. Healthy adult volunteers served as controls.

100 µl aliquots of heparinized whole blood were incubated on ice during 30 minutes with diluted antisera. These murine monoclonal sera were directly labeled with Fluorescine IsoThioCyanate (FITC) or phycoerythrin (PE) and added to a final dilution of 1:10 for anti CD64-FITC (Immunotech, Marseille, France) and 1:100 for all other monoclonals. These were anti-CD3-FITC with anti HLA-DR-PE, anti-CD4-FITC with anti-CD8-PE, anti-CD69-FITC with anti-CD3-PE, anti-CD20-FITC with anti-CD23-PE, and anti-CD14-PE (Becton Dickinson Immunochemistry systems, San Jose, CA). Samples were

then washed with phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA) and 0.02% NaAzide and after centrifugation at 1500 rpm red cells were lysed during 20'with 2 ml FACS Lysing Solution (Becton Dickinson Immunochemistry systems, San Jose, CA), centrifuged and resuspended twice in 0.01% Na-Azide in PBS. Immunofluorescence was measured with a FACS Calibur flowcytometer and analyzed with Cell Quest software (Becton Dickinson Immunochemistry systems, San Jose, CA). Cell populations were identified by scatter pattern (granulocytes vs. mononuclear cells). Subpopulations with a specific surface marker profile were expressed as percentage of mononuclear cells. Expression of CD 23 and CD 64 was measured as mean fluorescence intensity (MFI) of those markers on the (sub)population studied. To correct for inter-assay variability of MFI measurements, the MFI was expressed as the ratio of the value obtained in the patient sample and that obtained in a simultaneously tested sample of a healthy adult control.

Statistical analysis

Cell counts during and in-between fever attacks were compared by the Wilcoxon squared rank test, a 2-tailed p value of <0.05 being considered significant. Fluorescence intensity was similarly analyzed. Values are displayed as mean ± the standard deviation. All analyses were performed using GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego, CA)

Results

Patients

Six mevalonate kinase deficiency patients participated in the study. Five of these had the HIDS phenotype. These have been described previously, as Numbers 119, 132, 135, 137 and 139.(9). One child had the mevalonic aciduria phenotype.

In all patients mevalonate kinase deficiency has been established and mutations have been identified in both alleles of their MVK-genes. Four were compound heterozygous for the mutant alleles 1129 G>A (V377I) and 803 T>C (I268T), and one patient carried the V377I and 59 A>C (H20P) alleles. The mevalonic aciduria patient has been described elsewhere (chapter 7). He has only 0.12% residual mevalonate kinase activity, due to mutations 1000G>A and 421-422insG in his *MVK* genes.

White blood cell and differential counts were obtained during and in-between febrile episodes in all 6 patients. In five of these, these data could be obtained during 2 fever episodes and in 2 or 3 non-febrile intervals.

Immunocytochemical analysis was performed at least once during fever and once in-between attacks.

White blood cells

The fever attacks were characterized by leukocytosis which sometimes was extreme (>40x10^9/l). Leukocytes on average rose from 7.7x10^9/l between attacks to 18.9x10^9/l during fever (p=0.001).

Lymphoid cells

During fever attacks, the percentage of lymphocytes in the differential count decreased 4-fold. This was largely due to a rise in myeloid cells. Absolute lymphocyte numbers showed little decrease. (Table 1)

T-lymphocytes

Absolute T-cell numbers, as determined by CD3 expression were similar in-between and during fever attacks. There was a slight decrease of T-cells as a percentage of mononuclear cells during fever (Table 2). However, both absolute and percentual T-cell counts were normal for age (10).

CD4 and CD8 subsets were normal for age and remained stable during and in-between attacks (Table 2) as did the CD4 / CD8 ratio.

The proportion of T-cells expressing CD69, an early activation marker, in-between attacks (0.4-1.4%) was comparable to that observed by us in 41 healthy adult controls (1.1-1.4%). During fever there was a small increase in CD69 expression (Table. 2). The percentage of T-cells that expressed HLA-DR was very similar in-between and during fever and well within the normal range (10).

B-lymphocytes

The percentage of B-lymphocytes as determined by CD20 expression remained normal in-between and during fever episodes, as did absolute B-cell numbers (table 2). B-cell activation was assessed by the MFI of the Fc-

epsilon-Receptor II, CD23, on CD20 positive cells. B-cell activation between attacks was comparable to that in healthy adult controls. During fever there was no significant change.

Monocytes

Monocyte numbers rose 2.8-fold during fever attacks. This was not reflected in the monocyte percentage in the differential count, because of a concomitant rise in granulocytes (Table. 1). Activation of monocytes was assessed by the presence on the cell surface of CD64 (Fcgamma-Receptor I). Monocytes were identified by the expression of the lipopolysaccharide receptor CD14 (Figure 1a). Activation was quantitatively expressed as the ratio of MFI of CD64 on CD14- positive cells of patients over that on CD14- positive cells of healthy adult controls (Table. 2). During fever there was a 2.5-fold rise in CD64 expression on monocytes ($p=0.03$, Figure 1b).

Granulocytes

Neutrophil leukocytosis during attacks was striking (Table. 1) with absolute neutrophil counts rising over 4-fold ($p<0.001$). Band forms were present in a minority of patients during fever, but could be as high as 15%.Eosinophil counts decreased during fever (table 1).

CD64 expression was measured on granulocytes, i.e. on cells that had the light scattering characteristics of granulocytes on flow-cytometry (Figure 1c) and

were negative for CD14. The MFI of CD64 rose 6.4-fold during fever ($p<0.01$).

These changes in both number and degree of activation of monocytes and neutrophil granulocytes constituted the main abnormalities observed in mevalonate kinase deficient patients (Figure 1).

Discussion

Mevalonate kinase deficiency leads to recurrent bouts of generalized inflammation. The chain of events linking the metabolic defect to the inflammatory phenotype is understood incompletely. Somehow inflammatory effector mechanisms are activated. We aimed to determine in which cell population this occurred. Several leukocyte subsets, could be expected to be activated in this disorder. B-lymphocytes might be involved, as suggested by the polyclonal elevation of IgD and IgA, typical of HIDS(11). T-lymphocytes, had been implicated previously, because of the high serum concentrations of interferon-gamma during fever attacks(8). Cells of the monocyte/macrophage lineage were expected to be activated. Inflammatory mediators typically produced by such cells, have been found to be secreted in increased amounts

either in-vivo, ex-vivo or both(7,12). Also, the raised urinary neopterin excetion is indicative of activation of mononuclear phagocytes (7). Finally, neutrophil granulocytes could be involved, since granulocytosis is a well known feature of the fever attacks in mevalonate kinase deficiency(1).

Despite the small number of patients studied, our data indicate that it is mainly the non-specific immune system, that is activated during fever episodes.

We could not detect signs of B-cell activation. T cells were not increased in numbers, nor was there increase in HLA-DR expression. The increase in CD69 expression on T-lymphocytes, though statistically significant, was very modest. It's biological significance, therefore, remains uncertain.

In contrast, there was a 4-fold increase in the numbers neutrophil granulocytes and a 3-fold rise in monocyte numbers. Moreover, these cells were activated, as reflected by the raised expression of CD64.

It can not be excluded, that these phagocytic myeloid cells are activated indirectly by some other cell population. Blood sampling during attacks took place as soon as fever had become manifest, so any activation preceding the onset of fever would not have been detected. It is conceivable that T-lymphocytes are involved in the initiation of the fever episodes, since these attacks are often triggered by immunizations or infections and the serum concentration of T-cell derived cytokines is elevated in HIDS. Also, the activation of non-circulating cells, such as plasma cells (responsible for IgA ad IgD secretion) sessile macrophages or dendritic cells would not have been detected by the present study.

However, the analogy with the other hereditary periodic fever syndromes would favor a central role for granulocytes and monocytes. Like HIDS, these are genetically determined autoinflammatory diseases, i.e. disorders in which inflammation is prominent but neither infectious organisms nor auto-reactive lymphocytes or auto-antibodies are involved (13). In two of these, Familial Mediterranean Fever (FMF) and the Cold-Induced Auto-Inflammatory syndrome / Muckle-Wells syndrome complex, the affected gene is expressed exclusively in granulocytes and monocytes (14,15,16). In the third autoinflammatory disorder for which the gene defect is known, the autosomal dominant TNF-Receptor Associated Periodic Syndrome, the 55kD high-affinity receptor for Tumor Necrosis Factor-alpha, is mutated(17). This receptor is expressed on many cell types, but among leukocytes, it is present only on granulocytes and monocytes. We have observed that the deficiency of mevalonate kinase worsens during fever. Moreover, further impairment of isoprenoid biosynthesis does augment the secretion of IL-1β by mononuclear cells upon stimulation via T-cells (JF and SH, unpublished). The body of data, therefore favors a model in which, the initiation of an attack may involve T-cell activation. The consequent activation of mononuclear and, ultimately, polymorphonuclear phagocytes, however, runs out of control due to the metabolic defect. These cells, and the soluble mediators they produce, then give rise to the symptoms patients suffer during their attacks. It has been hypothesized that the periodic fever syndromes result from impaired apoptosis

of (18). However, whether impaired apoptosis is instrumental in the observed increase of monocytes and neutrophils in this study remains to be investigated.

Acknowledgements

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Table. I

White blood cell and differential counts in mevalonate kinase deficiency patients during and in-between fever attacks.

		non febrile	febrile	p*
n		14	11	
white blood cells	$x10^9/l$	7.65 (± 3.20)	18.93 (± 5.7)	0.001
neutrophil granulocytes§	$x10^9/l$	3.72 (± 3.03)	14.95 (± 5.97)	0.001
polymorphonuclear granulocytes	%	46.5 (± 20.2)	78.8 (± 9.1)	0.003
band forms	%	0	2.4 (± 4.4)	n.s.
eosinophil granulocytes	$x10^9/l$	0.12 (± 0.08)	0.02 (± 0.05)	0.004
"	%	1.8 (± 1.3)	0.2 (± 0.4)	0.002
monocytes	$x10^9/l$	0.38 (± 0.16)	1.07 (± 0.59)	0.001
"	%	5.5 (± 2.0)	5.9 (± 3.2)	n.s.
lymphocytes	$x10^9/l$	3.42 (± 1 1.78)	1.91 (± 0.82)	0.024
"	%	46.1 (± 19.4)	11.0 (± 6.1)	0.001

Values are means (± standard deviation)
* 2-tailed Wilcoxon squared rank test
§ sum of polymorphonuclear granulocytes and band forms

Table. II

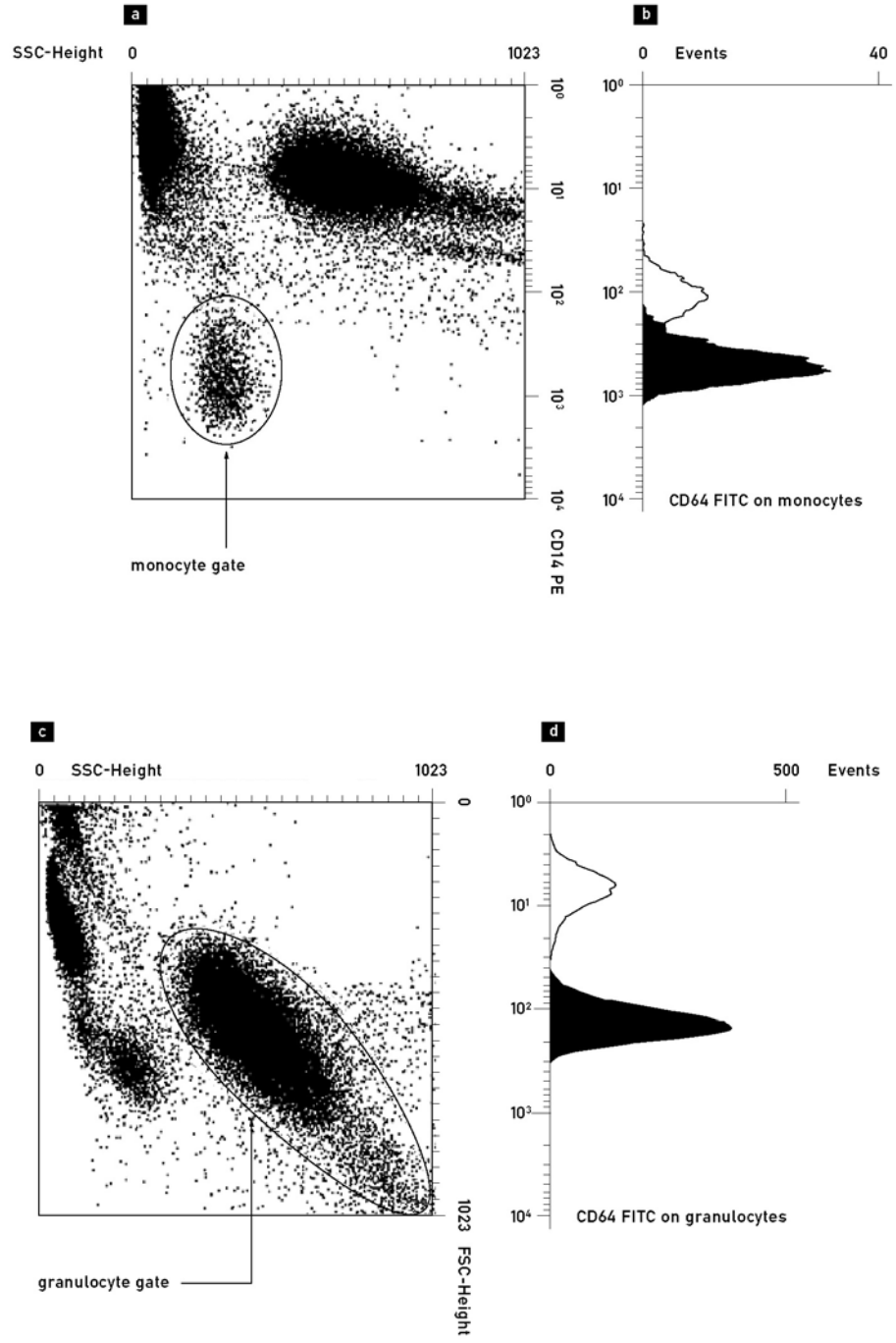
Immunocytochemical analysis of leukocytes of mevalonate kinase deficiency patients during and in-between fever attacks.

		non febrile	febrile	p*
n		10	9	
T-lymphocytes				
CD3	$x10^9/l$	2.38 (± 1.02)	2.09 (± 1.16)	n.s.
CD3	%§	74.2 (± 4.05)	61.1 (± 11.1)	0.008
CD4	%	39.8 (± 9.7)	40.8 (± 9.9)	n.s.
CD8	%	22.4 (± 9.1)	15.5 (± 6.9)	n.s.
HLA-DR on CD3+ve cells	%¶	5.2 (± 2.5)	4.0 (± 1.3)	n.s.
CD69 on CD3+ve cells	%¶	1.0 (± 0.5)	2.5 (± 0.9)	0.008
B-lymphocytes				
CD20	$x10^9/l$	0.26 (± 0.20)	0.58 (± 0.45)	n.s.
CD20	%	8.8 (± 4.2)	15.2 (± 9.5)	n.s.
MFI# of CD23 on CD20		0.80 9(± 0.32)	0.61 (± 0.45)	n.s.
myeloid cells				
MFI of CD64 on CD14+ve cells		1.33 (± 0.59)	3.30 (± 1.75)	0.031
MFI of CD64 on granulocytes		1.72 (± 0.99)	11.0 (± 11.8)	0.008

values are means (± standard deviation)
* 2-tailed Wilcoxon squared rank test
§ expressed as percentage of mononuclear cells
¶ percentage of CD3 positive cells
# MFI expressed as ratio over simultaneously tested healthy adult control

Legends

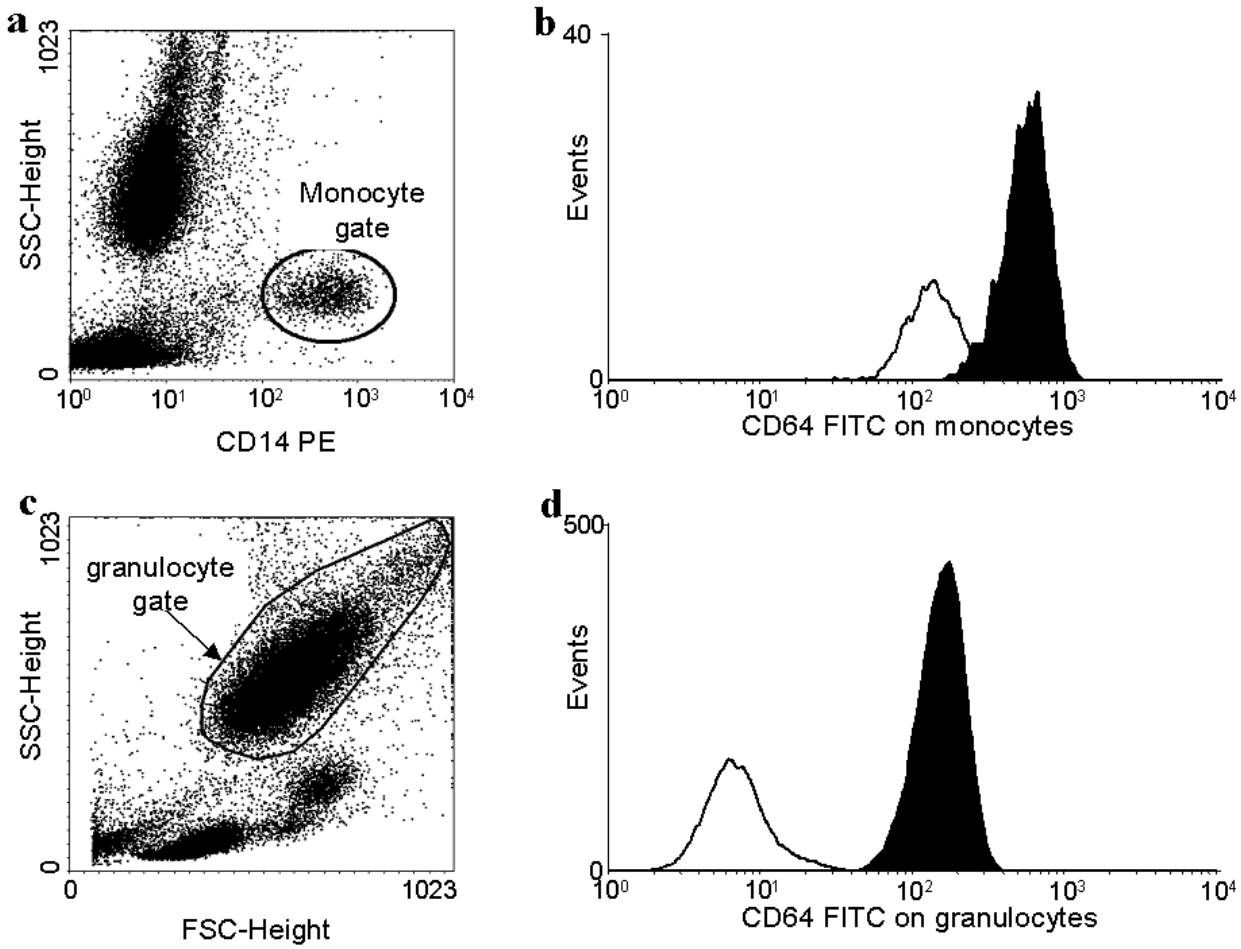
Figure 1



CD64 expression on granulocytes and monocytes.

Data are from a representative when febrile and in-between fever attacks. Panel a) Dot plot of forward scatter (side scatter (SSC). vs. immunofluorescence with anti CD14-PE. Analyses on monocytes were performed on cells within the indicated gate. Panel c) Dot plot of forward scatter (FSC) vs side scatter (SSC). Analyses on granulocytes were performed on cells within the indicated gate. Panels b) and d) CD64 fluorescence intensity on monocytes (panel b) and granulocytes (panel d) when febrile (black histograms) and in-between fever attacks (white histograms)

Figure 1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SSC-Height
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Monocyte
gate
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CD14 PE
b
40
Events
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10^0 10^1 10^2 10^3 10^4
CD64 FITC on monocytes
c
SSC-Height
1023
0
granulocyte
gate
0
1023
FSC-Height
d
500
Events
0
10^0 10^1 10^2 10^3 10^4
CD64 FITC on granulocytes

Increased urinary leukotriene E_4 during febrile attacks in the hyperimmunoglobulinaemia D and periodic fever syndrome.

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Abstract
Background:
The hyperimmunoglobulinaemia D and periodic fever syndrome is a hereditary periodic fever, caused by deficiency of the enzyme mevalonate kinase. It is unclear how this defect leads to recurrent fever episodes.
Aim:
To assess the involvement of cysteinyl leukotrienes in the pathogenesis of fever attacks as reflected by urinary leukotriene E4 (LTE_4) excretion.
Methods:
Urinary LTE_4 was measured in seven patients while febrile and afebrile
Results—LTE_4 was raised during fever in all subjects (46–199 nmol/mol creatinine, mean 92; normal <40). Urinary LTE_4 was normal between attacks, as well as in normal children with fever as a result of miscellaneous causes.
Conclusion:
Our results suggest that cysteinyl leukotrienes play a role in the pathophysiology of this disorder. As no effective treatment is yet available, leukotriene receptor antagonists might offer a new therapeutic approach for patients with the hyperimmunoglobulinaemia D and periodic fever syndrome.

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Keywords: cysteinyl leukotrienes; leukotriene E4; mevalonate kinase; periodic fever; immunoglobulin D

Dutch type periodic fever (MIM#260920), also known as the hyperimmunoglobulinaemia D syndrome (HIDS), is an autosomal recessive disorder, characterised by febrile attacks recurring at more or less regular intervals and the presence of an increased serum IgD concentration(1). Temperature typically rises abruptly, remaining continuously increased for three to six days. Febrile episodes recur every two to eight weeks from infancy. During these attacks patients often complain of malaise, chills, headache, arthralgias, nausea, vomiting, diarrhoea, and abdominal pain. The affected children often have striking cervical lymphadenopathy and may have splenomegaly, hepatomegaly, arthritis, and a variety of skin rashes (2). In the patient's blood, markers of inflammation such as white cell counts, erythrocyte sedimentation rate, and serum concentrations of several proinflammatory cytokines, are raised during febrile episodes (3), and the urine concentration of neopterin is acutely raised (4). Mutations in the *MVK* gene, which encodes mevalonate kinase, have been identified as the underlying genetic defect (5;6). These mutations lead to a substantially reduced activity of mevalonate kinase, a key enzyme in the biosynthesis of cholesterol and other isoprenoid compounds.
Another disorder, mevalonic aciduria7 (MA, MIM# 251170), has been described in which mutations in the *MVK* gene virtually abolish all enzyme activity of mevalonate kinase (7;8). This disease is characterised by failure to thrive, developmental delay, hypotonia, seizures, cerebellar ataxia, hepatosplenomegaly, anaemia, and a dysmorphic facies. Patients with MA suffer from recurrent attacks of generalised inflammation, similar to those observed in HIDS, but often more severe (9).
The cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) are endogenous lipid molecules derived from arachidonic acid through the 5-lipoxygenase pathway (10). These compounds are very potent proinflammatory mediators, acting at subnanomolar concentrations. Several cell types are capable of producing cysteinyl leukotrienes, including macrophages, eosinophils, and mast cells. Endogenous urinary LTE_4 excretion is a reliable index of the systemic production of cysteinyl leukotrienes in vivo (11). The pathogenesis of inflammation in both MA and HIDS remains unknown. However, in patients with mevalonic aciduria urinary excretion of LTE_4 was found to be highly increased , suggesting a pathogenic role for cysteinyl leukotrienes in MA (12;13). In the present study, we tested the hypothesis that cysteinyl leukotrienes are similarly involved in HIDS.

Patients and methods

After obtaining informed consent, urine samples were collected from seven children (aged 7–13 years), whose diagnosis of HIDS had been confirmed by demonstration of mutations in both copies of the *MVK* gene. From each case samples were obtained twice, once during a fever episode and once between attacks. Clinically, the fever could be attributed to an attack of HIDS (and not to an intercurrent infectious disease). Samples were collected in polypropylene containers and either immediately frozen and stored at -80°C (cases 1–3) or frozen at -20°C, thawed, transferred to smaller containers, and then stored at -80°C (cases 4–7) until analysis. The presence of pathological constituents was excluded.
Mevalonate concentrations were determined with a stable isotope dilution assay and gas chromatography–mass spectroscopy (5). Urinary LTE_4 was measured as described previously (11;14). Briefly, 3H- LTE_4 (Du Pont–New England Nuclear, Boston,

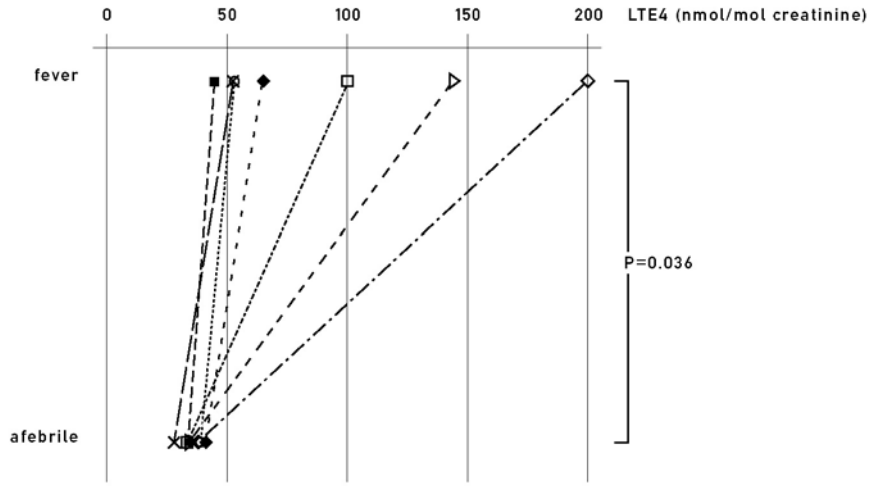
Massachusetts) was added as an internal standard. All urine samples were then acidified to pH 4.5 by addition of 0.1 mol/l HCl. Extraction of LTE_4 was performed on Sep-Pak C18 cartridges (Waters Associates, Milford, Massachusetts); separation of LTE_4 from other leukotrienes was carried out by reversed phase high pressure liquid chromatography. Quantification of LTE_4 was performed by enzyme immunoassays with specific antibodies (Cayman, Ann Arbor, Michigan). The corresponding specificity for the LTE_4 antibody was 100% for LTE_4, 10% for LTC_4, 9% for LTD_4, and <0.01% for LTB_4.

Statistical analysis was performed using a paired samples *t* test (SPSS 8.0).

Results

Between fever episodes the mean urinary LTE_4 excretion in HIDS patients was 36 (range 30–40) nmol/mol creatinine, well within the normal limit (<40 nmol/mol creatinine).

Figure 1



Urinary LTE_4 concentrations during and between fever episodes.

During fever episodes, urinary excretion of LTE_4 increased in all patients (mean 92, range 46–199 nmol/mol creatinine; fig 1). This was significantly higher than the values recorded when patients were afebrile ($p = 0.036$). As expected in HIDS, the concentrations of mevalonic acid in urine were increased during fever periods. However, there was no correlation between urinary concentration of mevalonic acid (4.2–41.5 mmol/mol creatinine; normal <0.1 mmol/mol creatinine) and that of LTE_4 (data not shown). In addition, urinary LTE_4 was found to be within normal limits (30.8 ±6.5) nmol/mol creatinine) in 20 infants and children with fever caused by viral or bacterial infections as well as fever of unknown origin, recruited from outpatients and inpatients at the University Children's Hospital, Heidelberg, Germany. Urinary LTE_4 remained unchanged in the same group of otherwise normal children when afebrile (31.4 ±6.3) nmol/mol creatinine).

Discussion

Cysteinyl leukotrienes have a pathophysiological role as mediators of inflammation in disease states like bronchial asthma, juvenile rheumatoid arthritis, or inflammatory bowel

disease(15;16). In addition, LTE_4 excretion was found to be enhanced in the most severe form of mevalonate kinase deficiency, MA (12). In this disorder and in contrast to HIDS, urinary LTE_4 is constantly raised, even though the highest values are found during febrile episodes. In this study, we showed increased excretion of LTE_4 in seven patients with the mild form of mevalonate kinase deficiency, HIDS, during fever, but not between attacks. The concentrations of LTE_4 during the attacks of cases 4–7 (open symbols in fig 1) were lower than those in the clinically equally severe cases 1–3 (closed symbols in fig 1), which in part might reflect the temporary thawing of urine samples in combination with the instability of LTE4. However, independently from the storage procedure, all urine samples from the patients with HIDS showed significantly raised LTE_4 concentrations during fever episodes.

Although the number of patients is small, our data point to a role of cysteinyl leukotrienes in the pathogenesis of the episodic inflammation in HIDS. While increased LTE_4 excretion is not specific for MA or HIDS, it is not merely a consequence of fever, as urinary LTE_4 is not raised in children with fever caused by viral or bacterial infections. The introduction of cysteinyl leukotriene receptor antagonists should help to elucidate the role of these mediators in HIDS. It is speculative whether such agents could reduce the severity of fever attacks in HIDS. As these have thus far been resistant to all current anti-inflammatory treatment (2), a
future new therapeutic approach would be most welcome.

Acknowledgements

We are indebted to J. de Jong, B.-T. Poll-The, M. Duran, and W. Kuis for their expert help and advice. This study was supported by a grant from the Deutsche Forschungsgemeinschaft, Bonn, Germany, to Dr Mayatepek (Ma1314/2–3).

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Temperature dependency of mevalonate kinase enzyme activity is a pathogenic factor in Hyper IgD and periodic fever syndrome

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submitted

Temperature dependency of mevalonate kinase enzyme activity is a pathogenic factor in Hyper IgD and periodic fever syndrome

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Abstract

Hyper IgD and periodic fever syndrome (HIDS) and mevalonic aciduria (MA) are autosomal recessive disorders characterized by recurrent episodes of fever and generalized inflammation. Both syndromes are caused by specific mutations in the gene encoding mevalonate kinase (MK), resulting in a depressed enzymatic activity mainly due to reduced protein levels. We studied the effect of temperature on the activity of wildtype and several mutant MKs in fibroblasts. All fibroblast cell lines originating from HIDS patients and harboring the common V377I *MVK* allele, displayed substantially higher MK activities at 30°C as compared to 37°C. As shown by temperature inactivation experiments this resulted in a protein nearly as stable as in control cell lines, indicating that it is primarily the maturation of the protein which is affected. Accordingly, when HIDS cell lines were cultured at 39°C, MK activity decreased further. This triggered a compensatory increase in 3-hydroxy-3-methylglutaryl-CoA reductase activity indicating that MK becomes progressively rate-limiting. A similar phenomenon occurs *in vivo*. MK activity in peripheral blood mononuclear cells drops 2-6-fold when HIDS patients experience febrile attacks. Thus, minor elevations in temperature, can set off a chain of events, with MK becoming progressively rate-limiting, leading to a temporary deficiency of isoprenoid end-products, followed by inflammation and fever.

Keywords: mevalonic acid, hypergammaglobulinemia, Immunoglobulin D, mutation, syndrome, inflammation, lipid metabolism

Introduction

Recurrent fever and generalized inflammation are the hallmarks of auto-inflammatory diseases, which include the hereditary periodic fever syndromes such as familial Mediterranean fever, TNF-receptor associated periodic syndromes, hyper IgD and periodic fever syndrome (HIDS, MIM 260920) and mevalonic aciduria (MA, MIM 251170)(1). HIDS and MA are relatively rare autosomal recessive diseases and are caused by a depressed activity of the enzyme mevalonate kinase (MK, EC 2.7.1.36)(2-4). In both MA and the more benign HIDS, patients suffer from recurrent fever episodes associated with lymphadenopathy, arthralgias, vomiting, diarrhea and skin rash (5;6). Fever episodes can be triggered by minor infections, physical and emotional stress, and childhood immunizations. However, most attacks occur without a clear precipitating event (5). Inflammation in MA may be more severe since it had been fatal in some cases (6). In addition to the fever episodes, MA patients have variable degrees of psychomotor retardation, facial dysmorphy, failure to thrive, hepatosplenomegaly, and anemia (5;6).

In MA, MK enzyme activity in patient cells is usually virtually undetectable (6;7). In HIDS, however, residual MK activity is measurable, and varies between 1% and 7% in cultured skin fibroblasts and lymphocytes (3;8). MK deficiency in both MA and HIDS is caused by mutations in the *MVK* gene which encodes MK (2-4;7-11). One particular missense mutation has been identified exclusively in HIDS. This mutation, 1129 G>A, leads to the substitution of the valine at position 377 for an isoleucine (V377I) and has been found in ~90% of HIDS patients analyzed so far (8;11). Most HIDS patients are compound heterozygotes for this mutation and a second missense mutation. The second mutation often is one that has been identified also in MA, implying that it results in a non-functional enzyme. This indicates that the V377I substitution is responsible for the HIDS phenotype.

It is unclear how mevalonate kinase deficiency leads to inflammation. Neither is it clear, why this inflammation is episodic rather than continuous. Previously, we and others demonstrated that heterologous expression of the V377I mutant MK protein in *Escherichia coli* yielded considerable residual enzyme activity (3;12). In contrast, cultured human skin fibroblasts homozygous for this mutation showed less than 8% residual enzyme activity. Furthermore, upon immunoblotting, hardly any MK protein could be detected (3), while Northern blot analysis demonstrated that this was not due to lowered *MVK* gene expression (8). Together, this suggested that the V377I mutation affects the stability and/or maturation of the mutant MK.

We now report that the common V377I mutation and a second mutation found in a patient with HIDS indeed affect the stability and/or maturation of MK in patient cells in a temperature sensitive manner. The decrease in MK activity occurring at elevated temperatures may provide an explanation for the episodic nature of the fever episodes in HIDS.

Patients and Methods

Patient cell lines and cells

After approval by the institutional ethical review board and written informed consent by the patients' parents, skin fibroblasts were obtained by skin biopsy and/or blood was drawn by venipuncture in sterile pyrogen-free heparinized plastic tubes. Peripheral blood mononuclear cells (PBMC) were isolated by ficoll hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation, washed twice with PBS, snap frozen in liquid nitrogen in aliquots of 3-10 x10^6 PBMC and stored at –80ºC until assay.

Fibroblast cell lines were cultured in Nutrient Mixture Ham's F-10 with L-glutamine and 25 mM HEPES (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Calf Serum (Gibco) at the temperatures indicated. For enzyme and immunoblot analysis cells were harvested and washed twice with phosphate buffered saline (PBS) after trypsinization, and either used directly or snap-frozen in liquid nitrogen and stored at –80ºC until use.

All patients and patient cell lines had low MK enzyme activity in combination with mutations in the encoding *MVK* gene.

MK and 3-hydroxy-3-methylglutaryl-CoA reductase enzyme analysis

MK activity was measured radiochemically in cell lysates using ^{14}C-labelled mevalonate (NEN, Perkin Elmer Life Sciences, Boston, MA)(13). 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) was measured radiochemically in cell lysates using ^{14}C-labelled 3-hydroxy-3-methylglutaryl-CoA (Amersham Pharmacia Biotech, HMG-CoA)(14). When necessary, the obtained batch of ^{14}C-HMG-CoA was further purified by cellulose thin layer chromatography using as solvent system 1-butanol : acetic acid : water (5 : 2 : 3). Cell pellets were lysed by sonication (twice, 40 J at 8 W output, with cooling between the pulse periods).

This was done in PBS when only MK was assayed or in HMGR assay buffer (14) when both HMGR and MK were measured simultaneously. In the latter case, extra $MgCl_2$ (12.7 mM instead of 6 mM) was added to assay MK in order to compensate for the EDTA and EGTA present in the HMGR assay buffer. The thermal inactivation experiments were performed in PBS containing 10 mM dithiothreitol (DTT) in order to prevent inactivation of MK due to oxidation (15). Samples were removed on time points indicated and assayed for activity. In every sample the activity of MK and/or HMGR was determined in duplicate.

Immunoblot analysis

Immunoblot analysis was performed in the same samples used for MK enzyme analysis. Antibodies were generated as described in Hogenboom et al. (14). Detection by chemiluminescence was performed using the Western Light system (Tropix, Applied Biosystems, Foster City, CA) or the enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech).

Statistical methods

The medians were compared by either an unpaired two tailed Mann-Whitney test, or a Kruskal-Wallis nonparametric ANOVA test followed by Dunn's multiple comparisons test. A p-value<0.05 was considered significant. The mean values are displayed ± the standard deviation.

Results

The V377I mutation affects MK activity in a temperature sensitive manner

Since episodic fever is a prominent symptom of HIDS and MA, we studied whether temperature has an effect on MK enzyme activity. To this end, skin fibroblast cell lines of control subjects, MA and HIDS patients with different genotypes were cultured at 30°C, 37°C or 40°C for 3 days. The HIDS cell lines used for this study were from patients which are compound heterozygous for the V377I and either the H20P mutation or the I268T mutation. The H20P mutation has been shown to result in an unstable and fully inactive protein and the I268T mutation in homozygous state results in MA (7;9). The MA cell lines were from patients which are homozygous for the I268T and for a V310M mutation and compound heterozygous for a H20P and an A334T mutation (7;9;10). Enzyme analysis in the various cell lines revealed that the residual MK enzyme activity in the HIDS cells cultured at 37°C varied between 1.1% and 3.0%, whereas the residual activity in the MA cells was virtually undetectable (figure 1). When the same cells were cultured at 30°C, the residual MK enzyme activity in the HIDS cells increased up to 9%, whereas the activity in these cells grown at 40°C became virtually undetectable. No change in the residual MK enzyme activities in the MA cells was detected. Although the MK activity in the control cells also increased at 30°C and decreased at 40°C, these differences were smaller than in the HIDS cell lines.

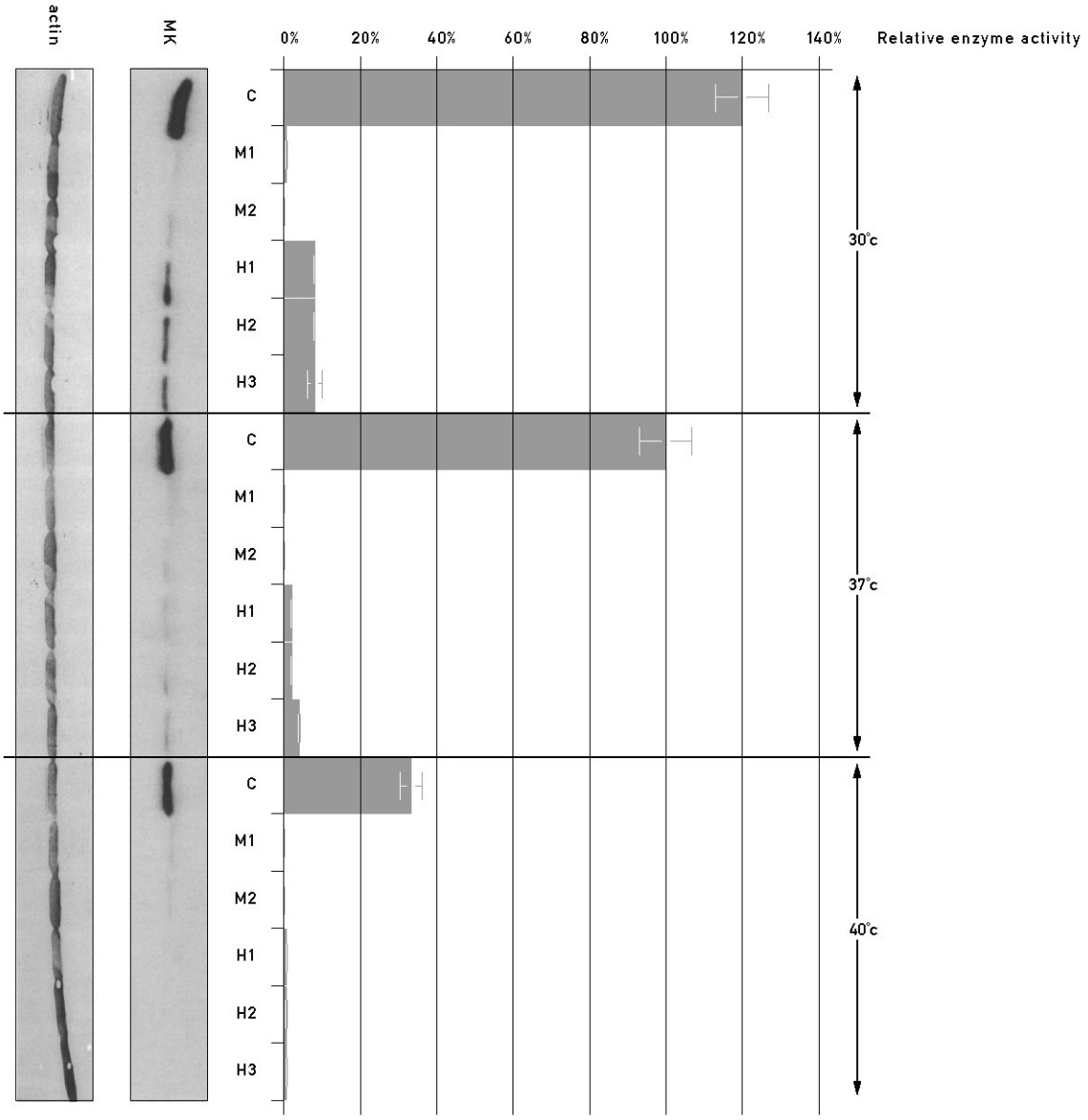

FIG. 1. Relative enzyme activities of MK in fibroblast lysates and immunoblot analysis of MK in the corresponding samples after 3 days of culture at the indicated temperature. The specific activity of the control fibroblast cell line grown at 37°C (598 pmol/min/mg protein) is used as the 100% value. For immunoblot analysis, equal amounts of protein (45 μg) were separated by 10% SDS-PAGE and analyzed on immunoblot with MK-specific antibodies. As a control for equal loading, the blot was analyzed with actin-specific antibodies. The figure includes a control cell line (C), two MA cell lines (M1, I268T; M2, V310M) and three HIDS cell lines (H1, H20P/V377I; H2, H20P/V377I; H3, H20P/V377I). The results for the HIDS and MA cell line with the I268T/V377I and H20P/A334T genotypes are not shown.

To determine whether the increase in MK activity at lower temperatures is due to an increase in MK protein levels we performed immunoblot analysis with an MK-specific antibody (figure 1). This revealed that immunoreactive material in lysates of the HIDS cells grown at 30°C becomes readily detectable, while hardly any MK protein is detected in HIDS cells grown at 37°C. When grown at 40°C immunoreactive material is undetectable (figure 1). MK protein levels in the MA cells are very low but detectable and do not change with temperature. Taken together, these results indicate that all mutations tested result in unstable proteins with very low steady-state levels of active and properly folded MK protein. In the case of the V377I allele, however, the levels appear to be dependent on the culturing temperature.

To study this phenomenon in more detail, we transferred control, HIDS (H20P, V377I) and MA (I268T) fibroblast cell lines from 37°C to 30°C and measured MK activity after 24, 48, 96 and 144 hours of culture. Again the activity of MK in the MA cell line remained virtually undetectable at all time points, whereas the activity in the HIDS cell line increased to even 45% after 144 hours (figure 2a). Since this HIDS cell line is heterozygous for the V377I allele and the fully inactive H20P allele, enzyme activity is expected to increase no further than 50%. These results imply that the V377I allele produces a fully stable protein at 30°C.

To study the stability of the matured enzymes, we performed thermal inactivation experiments with MK in control and HIDS fibroblast lysates at 50°C and 37°C. The inactivation process followed first order kinetics and was significantly faster in HIDS cell lysates at both temperatures, although the difference was only 1.3-fold ($p<0.05$, figure 2b and 2c). This shows that the mutant V377I protein, after it has reached the active and properly folded conformation, is almost as stable as the WT protein. Moreover, it shows that the V377I mutation primarily affects its maturation and not the stability per se.

Figure 2a

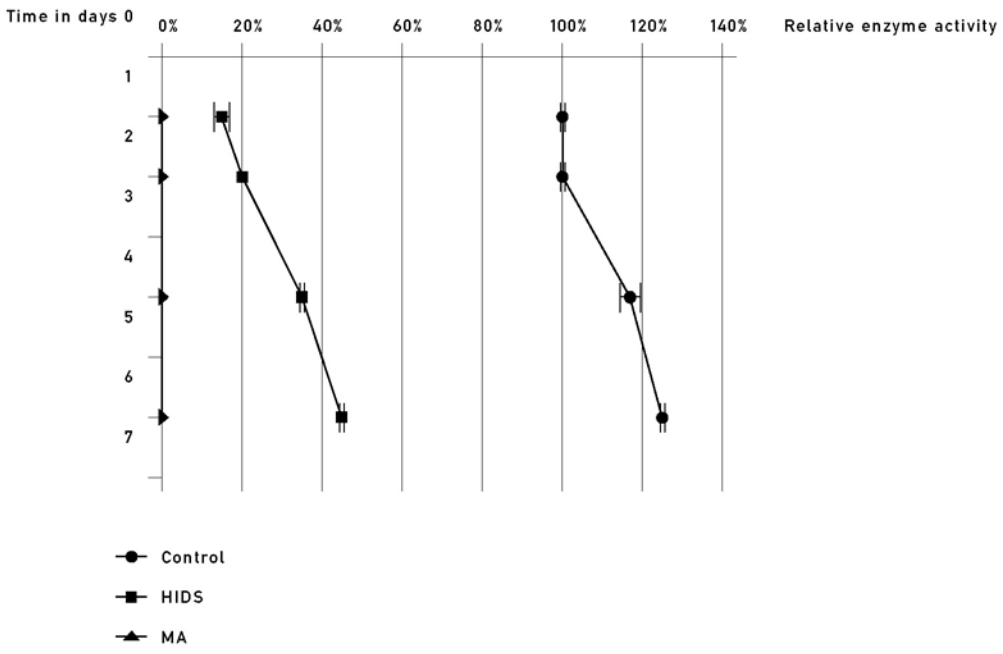

Figure 2b

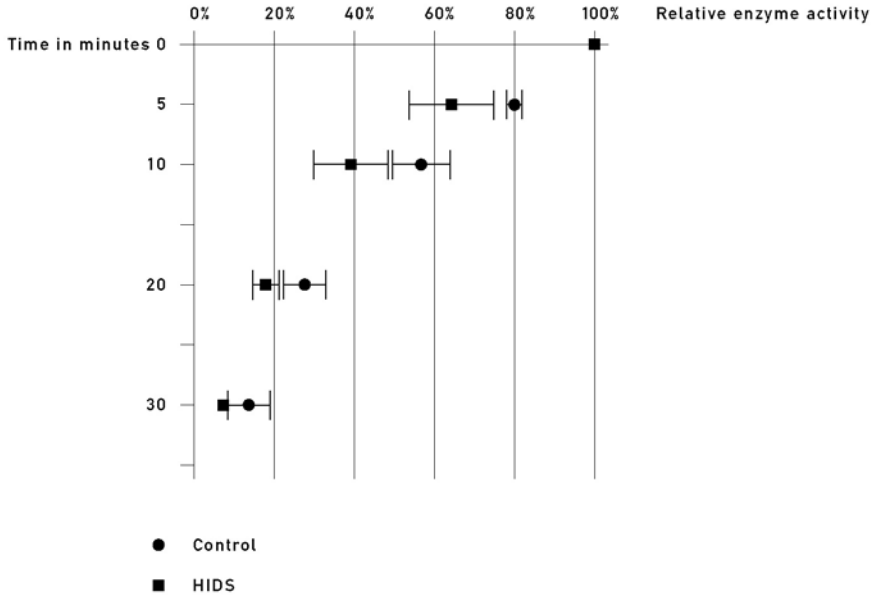

Figure 2c

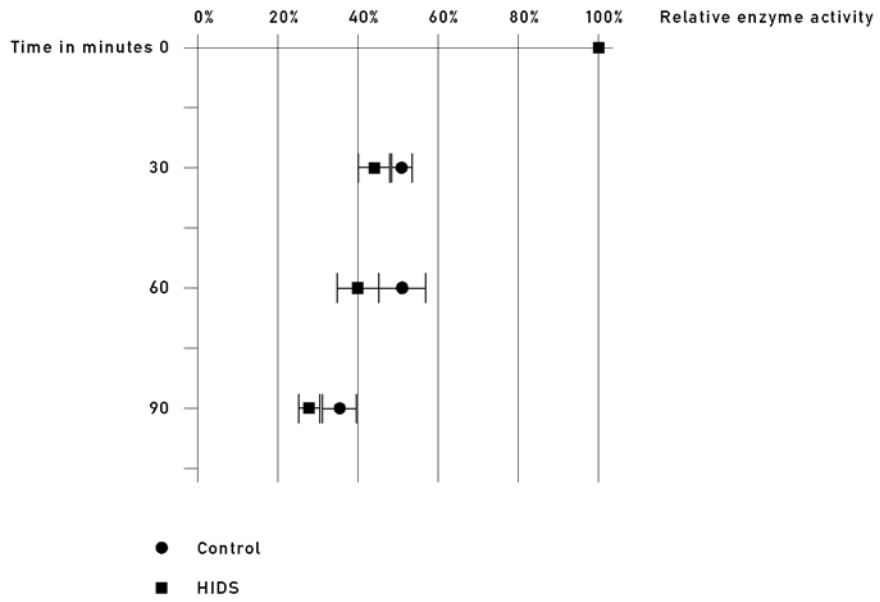

FIG. 2. **a**. Relative enzyme activities in fibroblast lysates of control, HIDS (H20P/V377I) and MA (I268T) cell lines after the indicated time grown at 30°C. The specific activity of the WT cell line after 1 day at 30°C (349 pmol/min/mg protein) is used as the 100% value. **b**. Thermal inactivation of MK enzyme activity in WT and HIDS cell lysates at 50°C. **c**. Thermal inactivation of MK enzyme activity in WT and HIDS cell lysates at 37°C.

Temperature sensitivity in another HIDS causing mutation

Although the vast majority of HIDS patients carry the V377I allele, at least three additional mutations appear specific for HIDS as well: A148T (442 G>A), P167L (500 C>T), and T209A (625 A>G)(2;3;8;16). In order to study whether temperature sensitivity of the MK protein could be a common characteristic for HIDS, we tested a cell line heterozygous for the A148T allele and the I268T allele (8). To this end, this cell line, a control cell line and a HIDS cell line containing the V377I allele were cultured at 30°C and 37°C for 6 days.

Residual activity of MK in the HIDS cell line containing the V377I increased 9-fold, and in the HIDS cell line with the A148T mutation the residual activity increased 8-fold, which indicates that the two mutant alleles produce MKs with similar temperature sensitivity (figure 3).

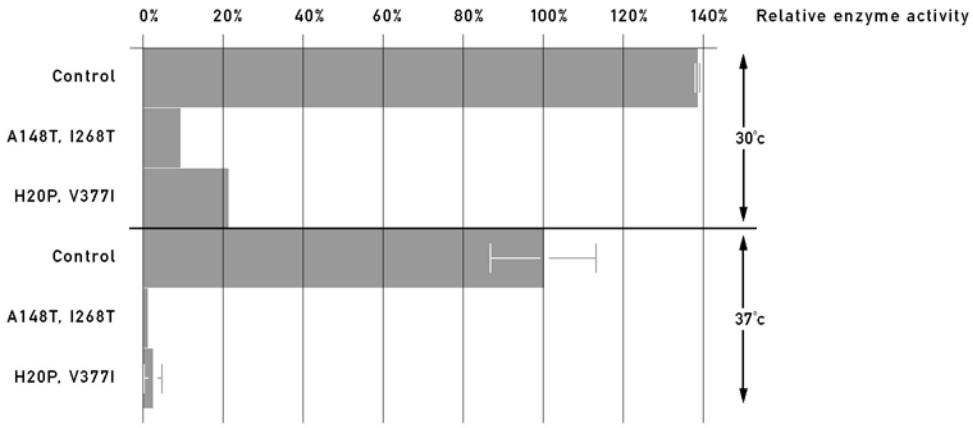

FIG. 3. Relative enzyme activities of one control, one HIDS cell line with V377I (H20P/V377I) and one HIDS cell line without V377I (A148T/I268T) after 6 days of culture at 30°C. The specific activity of the control fibroblast cell line grown at 37°C (543 pmol/min/mg protein) is used as the 100% value.

Temperature elevation induces increased HMGR activity in HIDS fibroblasts

Table 1. HMGR enzyme activity in fibroblasts and PBMCs from controls subjects, HIDS and MA patients.

	Fibroblasts	PBMCs
Controls	5±2 (24; 5)	2±1 (20; 10)
HIDS	7±4$^{\$}$ (19; 7)	12±4*** (15; 7)
MA	73±46*** (26; 5)	40 (1; 1)

Values are expressed as pmol/min.mg protein.
$^{\$}$p=0.5, ns
***p<0.001
The number of samples and different subjects, respectively, are displayed in parentheses.

HMGR is among the most tightly regulated enzymes in nature and is the rate-limiting enzyme step in isoprenoid biosynthesis (17). As previously reported by Gibson et al. (18), we observed an increased HMGR activity in cultured skin fibroblasts of MA patients (table 1). In fibroblasts of HIDS patients, however, HMGR activity was within the normal range. Since we found that MK activity is temperature-sensitive in HIDS cells, we investigated whether an increased temperature induces an increase in HMGR activity. Indeed, when switched to 39°C a marked difference between HIDS and control fibroblasts was observed (table 2).

Table 2. MK activity, HMGR activity and the MK/HMGR ratio in fibroblasts of control and HIDS patients cultured at different temperatures.

Subject	Condition	MK pmol/min.mg	HMGR pmol/min.mg	MK/HMGR
C1	37°C	394±14.9	6±0.1	64
C1	39°C, 6h	347±10.0	6±0.1	59
C1	39°C, 23h	313±2.4	7±0.4	43
C2	37°C	343±38.8	4±0.4	77
C2	39°C, 7h	311±11.2	4±0.5	87
C2	39°C, 25h	274±6.0	6±0.5	48
C2	37°C	357±20.0	6±1.6	57
C2	39°C, 7h	310±7.8	6±0.5	51
C2	39°C, 24h	288±2.4	11±1.0	27
H1	37°C	19±1.2	7±0.4	2,6
H1	39°C, 6h	9	12±1.6	0,72
H1	39°C, 23h	7±0.9	15±0.3	0,47
H2	37°C	22±0.8	8±0.5	2,7
H2	39°C, 6h	11±0.1	13±0.0	0,87
H2	39°C, 23h	10±0.3	26±2.2	0,39
H3	37°C	21±0.6	4±0.5	4.7
H3	39°C, 7h	11±0.2	4±0.5	2.4
H3	39°C, 25h	4±0.5	9±0.2	0.46
H4	37°C	44±0.6	10±1.0	4.3
H4	39°C, 7h	15±0.3	19±0.7	0.77
H4	39°C, 24h	22±0.9	13±0.3	1.62

The presented data in the table are derived from three independent experiments. The control cell lines are denoted as C, the HIDS cell lines as H1 (I268T/V377I), H2 (H20P/V377I), H3 (H20P/V377I) and H4 (I268T/V377I).

After 6 hours there was a decrease in MK activity and an increase in HMGR activity in most HIDS cells, whereas this did not occur in control cells. After 24 hours this effect was even

more pronounced, which is reflected clearly in the 4 to 7-fold decrease in MK/HMGR ratio (table 2).

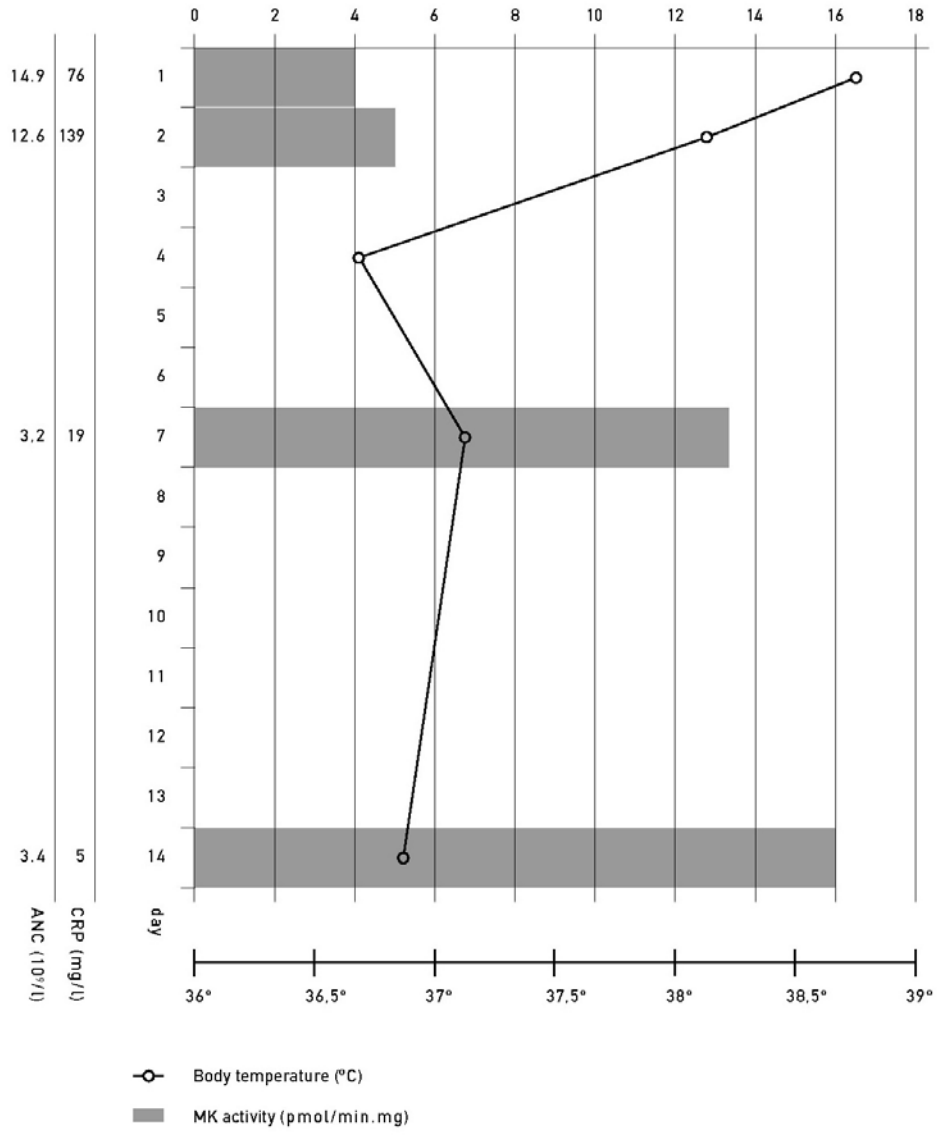

FIG. 4. A fever episode in a HIDS patient (I268T/V377I). The figure displays body temperature, MK activity, C-reactive protein (CRP) and absolute neutrophil count (ANC) at the indicated time-points during the fever episode.

Decrease of MK activity in HIDS PBMCs during fever

To investigate whether temperature also affects *in vivo* MK activity in patients, we analyzed MK and HMGR activity in PBMCs obtained from four HIDS patients during and between fever attacks. From HIDS patient A (table 3, figure 4), we obtained PBMCs on the first and second day of a fever attack and twice after recovery. The generalized inflammation during the fever attack was reflected by neutrophil leukocytosis and elevated C-reactive protein (CRP) levels. We measured MK and HMGR activity in all samples. As shown in figure 4, in this patient the MK activity of ~14 pmol/min.mg (6% of the control value) between fever episodes decreases 3-fold during fever (~5 pmol/min.mg). In patient B and patient C the drop in MK activity during fever was even higher: six- and seven-fold respectively. Finally, in one clinically severely affected patient, MK activity showed no response to the fever and remained low whether or not fever was present. Overall, in the 4 HIDS patients, MK activity during fever episodes was significantly lower than at normal temperature ($p<0.05$).

In general, the HMGR activities measured in the PBMCs from the HIDS patients appeared significantly elevated compared to controls, although less than in PBMCs of an MA patient. Analysis of the HMGR enzyme activity in the samples obtained from the four HIDS patients during and between fever attacks revealed a 2-fold rise in HMGR activity during fever in patients B and C, whereas in patients A and D there was only a modest response to fever or none at all. Thus, the patients who showed the most pronounced difference in MK activity also displayed the greatest difference in HMGR enzyme activity. Overall, however, the difference between HMGR activity during and between fever episodes was not significant ($p<0.1$).

Taken together, these results indicate that although MK activity is lowered at all times in PBMCs from HIDS patients, this deficiency becomes more prominent during fever.

Table 3. MK activity, HMGR activity and the MK/HMGR ratio in PBMCs of HIDS patients during and in between fever episodes.

Patient	Condition	MK pmol/min.mg	HMGR pmol/min.mg	MK/HMGR
A	Fever	5	12	0.38
A	Non febrile	14	15	0.95
B	Fever	1	20	0.07
B	Non febrile	6	8	0.74
C	Fever	1	16	0.07
C	Non febrile	7	9	0.82
D	Fever	2	14	0.12
D	Non febrile	3	9	0.31

The mean MK and HMGR values in controls are 217±51 and 2±1 pmol/min.mg, respectively. All patients had the same genotype, I268T/V377I.

Discussion

Three years after the identification of the genetic defect in HIDS, the pathogenesis of the fever attacks that characterize the disease remains elusive. It is not known how the metabolic defect leads to inflammation. Neither is it known why inflammation is episodic rather than continuous. Patients often report attacks after trivial events such as minor infections, vaccinations or vigorous exercise, events that also in otherwise healthy individuals temporarily raise body temperature. We hypothesized that such an increase in temperature may be involved in triggering the attacks. Since most *MVK* mutations result in deficient MK activity mainly due to reduced protein levels, we studied the effect of changes in temperature on the activity of normal and mutant mevalonate kinase.

We found that two *MVK* alleles exclusively associated with HIDS (V377I and A148T) display temperature sensitivity with respect to MK activity. For mutant alleles found in MA we did not observe such temperature sensitivity. When grown at 30°C the residual MK activity in cultured HIDS fibroblasts increases much more than in control cells, whereas at 40°C the residual activity diminishes. Importantly, this change in enzyme-activity correlates nicely with similar changes in the MK protein-levels as determined by immunoblotting. Since thermal inactivation of the mutant MK protein appears comparable with the WT protein, the observed low steady-state protein level in cells of HIDS patients is most probably due to inefficient or incorrect folding of the mutant protein at 37°C leading to its degradation. Recently, Ríos *et al* (12) characterized the V377I mutant protein expressed in *E. coli*. They found only modest kinetic differences in comparison with the WT enzyme (6-fold inflation of the K_m for mevalonate) and, as we did in patient fibroblast cell lines, normal thermal inactivation of the mutant protein. From these results these authors concluded that the V377I mutation is unlikely to provide an explanation for the observed depressed MK protein levels

and catalytic activity in HIDS. However, our results with the fibroblast lysates show that the V377I allele encodes a polypeptide that apparently is not capable of folding in the correct conformation at higher temperatures and subsequently undergoes degradation. These results together with all the genetic evidence such as the occurrence of this specific mutation in the vast majority of HIDS patients (8;11), its absence in control subjects (2), the results of linkage analysis (2) and the autosomal recessive mode of inheritance, unequivocally demonstrate that the V377I allele is the cause of the depressed MK activity in HIDS.

When we measured HMGR enzyme activity in cultured HIDS and MA fibroblasts, we found the activity elevated in MA fibroblasts as previously reported by Gibson at al. (18), whereas in HIDS cells, it was within the normal range. These results indicate that under the tested culture conditions MA cells compensate for their reduced MK activity, whereas in HIDS cells no compensation occurs. When HIDS fibroblasts are cultured at 39°C, however, the decrease in the enzymatic activity of MK is associated with an increased HMGR activity, in contrast to the control cells. This indicates that due to the increase in culture temperature MK becomes progressively rate-limiting, resulting in an increased HMGR enzyme activity in order to compensate for the decrease in MK activity. These results thus provide an explanation for the observed increase in mevalonate excretion during fever episodes in HIDS (19). Together with the reported difference in mevalonate excretion in urine between HIDS and MA (3;6;19), these observations also imply that in MA, MK performs the rate-limiting step instead of HMGR, whereas in HIDS the control of the pathway, at least at normal temperatures, remains at the level of HMGR.

We investigated whether the thermosensitivity of MK activity observed *in vitro* also plays a role in HIDS patients during and between fever episodes. Therefore we analyzed MK and HMGR activity in PBMCs obtained from four HIDS patients during and between fever attacks. We observed that MK enzyme activity drops 2-6-fold when HIDS patients experience

febrile attacks. In contrast to the normal HMGR enzyme activity in fibroblasts from HIDS patients, we observed an elevated HMGR enzyme activity in PBMCs from these patients. Although this elevation was not as high as in a sample obtained from an MA patient, it indicates that there is a derangement in isoprenoid biosynthesis in PBMCs from HIDS patients. No significant difference was observed in HMGR enzyme activity during and between a fever episode. However, the two patients who showed the most pronounced difference in MK activity also displayed an additional elevation in HMGR enzyme activity during fever. This could suggest that an elevation in HMGR enzyme activity may be missed in the other patients due to a delay between the onset of the attack and sampling. Our results show that during fever, MK deficiency in PBMCs from HIDS patients becomes even more prominent. From our *in vitro* data we conclude that this is caused by the impaired maturation of MK especially at higher temperatures.

There are several indications that isoprenoid biosynthesis plays a role in inflammation. For example, it has been reported that administration of LPS, TNF-α, or IL-1β to Syrian hamsters triggers a rapid upregulation of hepatic HMGR and a downregulation of squalene synthase, the enzyme catalyzing the first committed enzyme step of sterol biosynthesis (20-22). These observations suggest a higher demand for non-sterol isoprenoids during inflammation. Examples of non-sterol isoprenoids are the farnesyl- and geranylgeranyl-groups used for protein isoprenylation, isopentenyl tRNAs, dolichol, ubiquinone-10, and heme A. Furthermore, statins, a class of lipid-lowering drugs that are competitive inhibitors of HMGR and accordingly lead to decreased production of isoprenoids, have been reported to have both anti-inflammatory (23-26) and pro-inflammatory effects (27-29). Our data reported here suggest that in HIDS patients, the fever which accompanies inflammation enhances the MK deficiency, which will result in a temporary reduction in non-sterol isoprenoid production. This will affect especially isoprenoids with a high turnover, such as ubiquinone-10 in plasma,

which is decreased in most MA patients(30), prenylated small G-proteins like Rho (31), which are involved in multiple cellular processes like signal transduction or cytoskeletal organization, and the prenylated guanylate-binding proteins (GBP) which are specifically synthesized in response to IFN-γ and LPS (32). It is conceivable that this shortage of non-sterol isoprenoids is responsible for the pro-inflammatory phenotype of HIDS and MA. Thus, even minor elevations in temperature, due to exercise or infections could set off a chain of events, with MK becoming progressively rate limiting, leading to a temporary deficiency of anti-inflammatory isoprenoids, followed by inflammation and fever.

Strong support for this hypothesis is provided by the negative outcome of a therapeutic trial in which two MA patients were treated with low doses of lovastatin in order to block the production of mevalonate (speculated to be pathogenic). This trial had to be stopped because of the development of severe clinical crises (6). The outcome of this trial strongly suggests that the symptoms of MA are not caused by an excess of mevalonate but by a shortage of isoprenoid endproducts. The enormous difference in urinary mevalonate excretion in HIDS and MA patients also argues against a causative role of mevalonate in the pathogenesis of the clinical crises. MA patients have much higher mevalonate levels, but fever episodes occur as frequently as in HIDS.

The observed differences in stability and temperature sensitivity of mutant MKs may explain why HIDS patients display episodic fever. Since such fever episodes are prominent not only in HIDS but also in MA, the same may be true for MA. Indeed, the fact that mevalonate excretion in urine of both HIDS and MA patients correlates with disease severity points to a similar mechanism (6;19). Unfortunately, the extremely low and already hardly detectable MK activity levels in MA cells do not allow to demonstrate a similar temperature sensitive phenomenon. However, the finding that the increase in temperature does not only affect the mutant MKs, but also the wild-type MK activity makes it highly plausible. Even a small

additional decrease in the already extremely low MK activity in MA cells may have far reaching consequences. Since HMGR already appears maximally induced to compensate for the MK deficiency, a further induction to establish even higher mevalonate levels may not be possible. Furthermore, in MA cells the MK enzyme will be saturated already, thus an additional increase in the mevalonate concentration will have no effect on the flux through the pathway. These reasons may provide an explanation for the reported fatal outcome of a fever episode in several MA patients (6).

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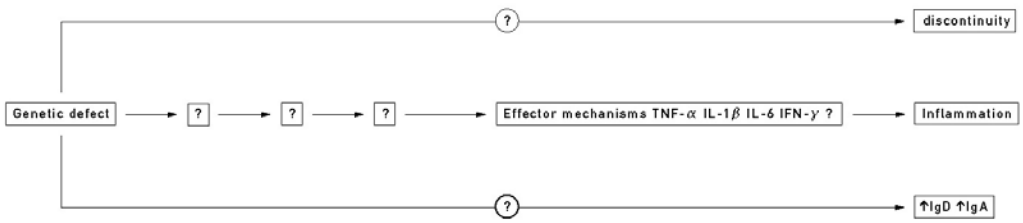
Summary and general discussion

Summary and general discussion

Since its initial recognition by van der Meer nearly 20 years ago(1), much has been learned about the Hyper IgD and Periodic fever syndrome (HIDS) or Dutch periodic fever (DPF) (MIM#260920). The clinical features of the disease have been analyzed in detail (2). The three cardinal features of the syndrome, the raised IgD and IgA, the generalized inflammation, leading to high fever, and the discontinuity of clinical symptoms remained largely unexplained and effective treatment has remained elusive. Studies into the inflammatory effector mechanisms involved, identified increased activity of pro-inflammatory cytokines, such as Interferon (IFN)-γ, Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-1β and IL-6 (3-7).

Figure 1

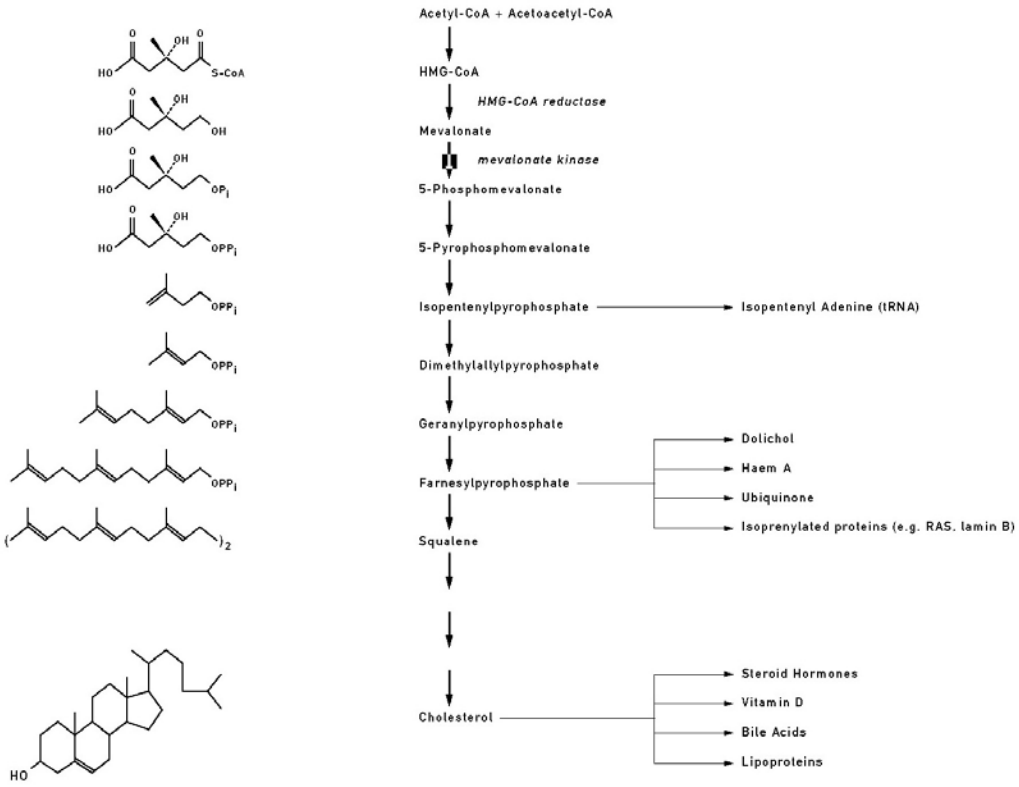


Understanding of the pathogenesis of HIDS in 1999

In 1999, it became clear that mevalonate kinase deficiency could lead to HIDS (8;9). The unexpected finding of an inborn error of metabolism at the root of this inflammatory disease generated at least as many questions as answers (fig. 1)(10). To name just a few, what exactly is the relation between mevalonate kinase deficiency and HIDS?

- Do all patients diagnosed with HIDS have mevalonate kinase deficiency?
- What causes some cases of mevalonate kinase deficiency to present with the HIDS phenotype and others with the more severe mevalonic aciduria phenotype?
- How does mevalonate kinase deficiency give rise to inflammation? Is this due to excess of its substrate (mevalonate) or shortage of one of the many products of isoprenoid biosynthesis (fig.2)?
- In which cells or which tissue(s) does this metabolic deregulation have its inflammatory effect?
- What other inflammatory effector mechanisms are involved?
- And how can we explain that a continuous metabolic defect leads to episodic disease. These were the questions that we attempted to address in this study.

Figure 2



Schematic representation of isoprenoid biosynthesis pathway.

In **chapter 2**, we studied a group of children with HIDS(11). It turned out that not all HIDS patients have mevalonate kinase deficiency. Indeed, entirely normal mevalonate kinase activities were found in about 20% of all patients. This finding has now been confirmed in adult patients as well (12). Hence, HIDS is a phenotype of mevalonate kinase deficiency, but patients with the HIDS phenotype, without the mutant *MVK* genotype, are not rare. However, some features, such as onset in infancy, prominent skin rash and diarrhea, appear more common in the DPF- or true HIDS-patients, i.e. those with mevalonate kinase deficiency. Elevated serum IgD has been reported to occur in all the other known hereditary periodic fever syndromes, as well

as in the periodic fever- aphtous stomatitis pharyngitis adenitis (PFAPA) syndrome (13-15). It might well be present in other, as yet unrecognized periodic fever syndromes. However, what is unusual about the DPF or true HIDS, as it has been called, is that hyperimmunoglobulinemia D is so extremely common. It is presently unknown how this rise in IgD is caused. Eventual unraveling of its mechanism should provide valuable insight in the relation between isoprenoid biosynthesis and the immune system. The physiological function of serum IgD is not known nor is its role in HIDS (16). However, HIDS patients may initially have normal serum IgD values (17), and in some patients IgD will remain normal throughout (8). High IgD, therefore, is unlikely to have an important role in the causation of inflammation. The finding of mevalonate kinase deficient patients with normal IgD and of HIDS patients with normal mevalonate kinase creates a semantic problem. The name by which the disease is identified in the On-line Mendelian Inheritance In Man (OMIM), Dutch type Periodic Fever, obviates this problem. However, this ignores the fact that many patients are not of Dutch descent (18). The nomenclature, therefore, remains a practical issue that needs to be solved.

Not all patients with mevalonate kinase deficiency present with HIDS. So much already followed from the finding of patients who were clinically and biochemically indistinguishable from HIDS, but had normal serum IgD (8). Furthermore there are two phenotypes of mevalonate kinase deficiency: HIDS and Mevalonic Aciduria. The latter is akin to HIDS in that patients suffer from recurrent inflammatory attacks. In addition, affected children may have various other problems. Commonly, MA patients are born small for gestational age. They grow poorly and their neurological development is usually impaired. Moreover their faces often show dysmorphic features (19). In **chapter 3** we show that unique mutations are involved in HIDS as

opposed to MA (20). All HIDS patients studied, had at least one copy of the *MVK*-gene that carried the 1129 G>A mutation coding for the amino acid substitution V377I. None of the MA patients studied had this mutation. The V377I mutant protein leads to a less profound MK deficiency than the mutations encountered in MA. It is this mild MK deficiency that appears to give rise to the HIDS phenotype. The distinction between the MA phenotype and the HIDS phenotype may however be less absolute than suggested here. In one family with HIDS, the two affected members (No's 15 and 16 in the international HIDS registry) are severely mentally retarded, despite being compound heterozygote for the mutations V377I and W62X and having 1.4% residual MK activity. Moreover, high IgD has been described in a mevalonic aciduria patient(21), indicating that the HIDS and MA phenotypes are the extremes of a continuous clinical spectrum. The rarity of patients, homozygous for V377I was striking in face of the frequency of this allele in compound heterozygotes. This suggests that individuals homozygous for V377I, are not being identified. Theoretically this could be due to intrauterine death of V377I homozygotes. This, however, is unlikely, since their defect is presumably milder than that in the compound heterozygotes who do in fact come to our attention. More likely, the V377I/V377I phenotype may in many instances be very mild or even asymptomatic. The genotype-phenotype relation in HIDS and mevalonate kinase deficiency, therefore, remains complex (fig.3).

Figure 3

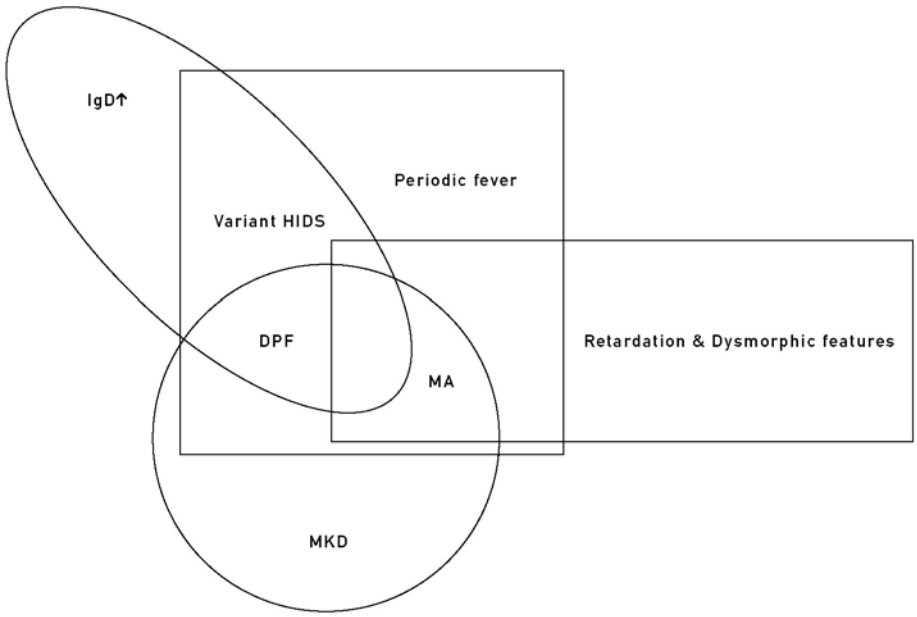


Diagram of the disease entities relevant to mevalonate kinase deficiency and periodic fever. MA: mevalonic aciduria, HIDS: HyperIgD and periodic fever Syndrome, DPF: Dutch type Periodic Fever, MKD: mevalonate kinase deficiency.

This complex genotype-phenotype relation inevitably has consequences for the diagnostic approach of the child with unexplained recurrent fever in general and of periodic fever due to mevalonate kinase deficiency in particular. The gold standard for the latter diagnosis is the demonstration of disease causing mutations in both *MVK*-alleles. When new mutations are found, measuring MK activity will be necessary to distinguish between true mutations and polymorphisms. Several diagnostic tests have been employed to diagnose true HIDS, notably, the serum IgD concentration and the urinary excretion of mevalonic acid. However, the performance of these test, i.e. their positive and negative predictive value, has not been formally evaluated. Preliminary data indicate, that, when first evaluated, less than half of the MK deficient children in our hospital had a serum IgD level exceeding the adult upper

limit of normal, 100 IU/ml. Age specific normal values for serum IgD have been reported to lead to an increased sensitivity (22). However, when applied to our population, the initial IgD measurement would have been elevated in only 45 % of MK deficient children. On repeated testing, this figure eventually does rise to 85%. The specificity of a repeatedly elevated serum IgD concentration does appear to be better with the application of age specific reference values instead of those for adults. However, these pediatric normal values have been collected in healthy Icelandic children, whereas, in clinical practice, the patient to be investigated has recurrent fever and is not Icelandic, for which case normal values are not available. Another problem is the interpretation of the urinary excretion of mevalonic acid. Mild elevations of urinary mevalonic acid and the corresponding lactone have been observed in all patients with mevalonate kinase deficiency (8;23;24). However, especially between febrile attacks, these levels may overlap with those observed in pediatric patients with children with inflammatory disorders and normal mevalonate kinase activities. Apparently, the normal values, obtained in healthy persons do not fully apply to these children. Clearly, additional studies are required to establish the value of urinary mevalonic acid measurement as a tool for the diagnosis of HIDS Measuring mevalonate kinase activity in patients' cells is feasible in expert laboratories. False positive results (i.e. deficient MK activities) have been obtained when studying leukocytes, whereas this is less likely to happen in cultured skin fibroblasts (Sander M.Houten, personal observation)

We therefore suggest that in children with a history, typical of HIDS, including onset in infancy, fever following vaccinations, lymphadenopathy and skin rash, genetic testing for *MVK*-mutations is performed, irrespective of serum IgD values. When feasible, this should be preceded by the assay of MK enzyme activity. In patients with

a less typical clinical presentation, the repeated finding of serum IgD over the 95th percentile for age should prompt *MVK* mutation analysis, again, preferably in conjunction with MK enzyme activity measurement.

This approach can teach us that periodic fever in an individual patient is due to mevalonate kinase deficiency. However, how this defect leads to fever remains elusive. Mevalonate kinase deficiency leads both to reduced production of isoprenoids (fig.2) and accumulation of mevalonic acid. The question which of these two phenomena gives rise to inflammation is addressed in **chapter 4**. In an ex-vivo model, we measured IL-1β secretion by isolated mononuclear cells in response to T-cell stimuli. In this model, reducing isoprenoid production led to a rise in IL-1β secretion, whereas the addition of mevalonate had no such effect. Similar observations have now been made by Kiener et al., when studying isolated normal monocytes treated with lovastatin and other lipophilic statin drugs (25). Therefore, it is unlikely that excess mevalonate is responsible for the inflammation in HIDS. Furthermore, the IL-1β secretion by HIDS cells could be reduced by the addition of farnesol, a precursor of isoprenoid synthesis, downstream of the defect in mevalonate kinase. Interestingly, varying the availability of mevalonate to cells from HIDS patients, had a profound effect on the IL-1β secretion by these cells. When mevalonate was reduced through the action of lovastatin, IL-1β secretion rose, whereas the addition of mevalonate led to a decrease in IL-1β secretion. These findings are in keeping with those of Houten et al, who demonstrated that protein isoprenylation in fibroblasts from HIDS patients was uniquely dependent on the availability of mevalonate. It follows that treatment of mevalonate kinase deficiency should focus on the restoration of isoprenoid biosynthesis, rather than on the inhibition of mevalonate production. If inflammation indeed results from a shortage of one or more of the many products of isoprenoid

biosynthesis, it still is unclear which product it could be and how it would normally act. Cholesterol and its derivatives are unlikely candidates, since inflammation is no feature of the inborn errors affecting the terminal steps of cholesterol biosynthesis (24). Furthermore, the studies performed in isolated monocytes by Kiener et al implicated shortage of non-sterol isoprenoids in the secretion of pro-inflammatory cytokines(25). Non-sterol isoprenoids are compounds such as presqualene diphosphate and a number of isoprenoid groups, which are normally attached to specific macromolecules. These are the isopentenyl groups of isopentenyl t-RNA's, dolichol, the polyisoprene groups of Heme-A and ubiquinone-10, and the geranylgeranyl- and farnesyl-groups, involved in post-translational protein modification. Examples of proteins that are geranylgeranylated are the Rho, Rac and Cdc42 molecules, involved in leukocyte activation and migration and the Rab molecules, involved in vesicle transport and exocytosis. The best known farnesylated proteins are molecules from the Ras- family, involved in cell proliferation. Whichever of these compounds is deficient in HIDS, any attempted therapy should aim at restoration of this deficit. To this end, two approaches could be envisaged. The first would be to increase the availability of mevalonate through up regulation of 3-hydroxy-3-methylglutaryl- (HMG-)CoA reductase. The synthesis of HMG-CoA reductase mRNA and the half life of the HMG-CoA reductase protein is known to increase in response to lowering intracellular cholesterol (26). This could be accomplished by elimination of dietary cholesterol and by means of treatment with oral bile acid sequestrant resins. These drugs deplete the bile acid pool. Consequently, cellular cholesterol is siphoned off into bile acid biosynthesis. The resulting increase in HMG-CoA reductase activity will restore cholesterol biosynthesis. It is, however, uncertain at best, whether it would lead to the restoration of non-sterol isoprenoid

levels. The second approach would entail the suppletion of mevalonate or isoprenoid intermediary metabolites, such as isopentenyl pyrophosphate, geranyl pyrophosphate, farnesyl pyrophosphate. The corresponding fatty alcohols might be useful pro-drugs in this respect. However, there are safety concerns regarding both approaches. Extreme cholesterol depletion, as would result from the combination of mevalonate kinase deficiency, dietary restriction and draining the bile acid pool, might theoretically carry the risk of myopathy or even rhabdomyolysis, since myopathy is a feature of both mevalonic aciduria and statin drug toxicity. As for treatment with isoprenoid fatty alcohols, there is no clinical experience whatsoever with these compounds. Indeed, feeding metabolites into a pathway that is normally very tightly regulated could have many untoward effects. Therefore these approaches should be tested in the animal model first. To this end, the generation, by site directed mutagenesis, of mevalonate kinase deficient mice is vital.

From the previous it appears that decreased biosynthesis of non-sterol isoprenoids and inflammation are linked. Non-sterol isoprenoids are essentially intracellular molecules. Hence, there must be cells in which the disturbed isoprenoid biosynthesis leads to loss of control of inflammatory activation of that same cell. We therefore had to know, which cell population was activated in HIDS.

In **chapter 5** we studied leukocytes from HIDS patients during and between fever attacks. Several leukocyte subsets, could be expected to be activated in this disorder. B-lymphocytes might be involved, as suggested by the polyclonal elevation of IgD and IgA, typical of HIDS (27). T-lymphocytes, had been implicated previously, because of the high serum concentrations of IFN-γ during fever attacks (5). Cells of the monocyte/macrophage lineage were expected to be activated. Inflammatory

mediators typically produced by such cells, have been found to be secreted in increased amounts either in-vivo, ex-vivo or both (6;7). Also, the raised urinary neopterin (7) and Leukotriene E_4 excretion (chapter 5) is indicative of activation of mononuclear phagocytes. Finally, neutrophil granulocytes could be involved, since granulocytosis is a well known feature of the fever attacks in mevalonate kinase deficiency (2). Though discrete signs of T-cell activation were detectable, the most striking changes were observed among myeloid cells. There was a 4-fold increase in the number of neutrophil granulocytes and a 3-fold rise in monocyte numbers. Moreover, these cells were activated, as reflected by the raised expression of CD64. It can not be excluded, that these myeloid cells are activated indirectly by some other cell population. It is conceivable that T-lymphocytes are involved in the initiation of the fever episodes, since these attacks are often triggered by immunizations or infections and the serum concentration of T-cell derived cytokines is elevated in HIDS. Also, the activation of non-circulating cells, such as sessile macrophages or dendritic cells would not have been detected by the present study, whereas the enlargement of lymph nodes, liver and spleen during fever attacks in HIDS would be compatible with the involvement of such cells.

However, the analogy with the other hereditary periodic fever syndromes would favor a central role for granulocytes and monocytes. We speculate that it is in these myeloid cells, that the metabolic defect interferes with some, as yet unidentified, anti-inflammatory pathway. This anti inflammatory pathway might involve the apoptosis of activated granulocytes and monocytes, since the genes mutated in hereditary periodic fever syndromes other than HIDS all encode proteins presumed to play a role in apoptosis (28). The sequence of events would then be, that these cells are triggered, e.g. via T-cell activation, after which inflammatory activation runs out of control.

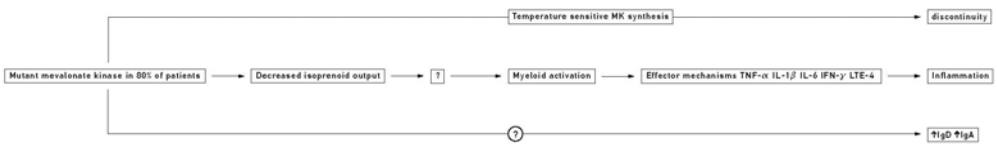
From the point of view of treatment, given the limitations of approaches aimed at increasing isoprenoid biosynthesis, non-specific inhibition of neutrophil and monocyte/macrophage activation would be an attractive option. However, experience with drugs that act in this fashion has been disappointing. Glucocorticoids, colchicine and thalidomide have all met with limited success (2;29). Biological agents, interfering with the soluble mediators produce by monocytes/macrophages do hold promise, however. Limited experience with etanercept, a fusion protein of the Fcγ and the ligand binding domains of the TNF receptor TNFRSF1B, has been encouraging. Similar "biologicals" have been developed which antagonize the actions of IL-1 (IL-1-receptor antagonist, anakinra) or IL-6(30;31). Future studies will have to determine whether such agents have a place in the prophylaxis or treatment of fever attacks in HIDS.

In **chapter 6** an other potential target for treatment was identified. Having identified MK deficiency as the cause of HIDS, we studied whether cysteinyl leukotrienes, known to be involved in the fever attacks of MA, were also involved in HIDS. From the urinary excretion of LTE_4 it appeared that cysteinyl leukotrienes were indeed overproduced in HIDS, but only during fever attacks. The source of these lipid mediators was not studied, but given the data presented in chapter 4, mononuclear phagocytes could well be involved. LTE_4 excretion has been described in other severe inflammatory states, such as Kawasaki's disease (32), and is by no means specific for HIDS. It is to be regarded as one of the many effector mechanisms reflecting uncontrolled macrophage activation. However, cysteinyl leukotrienes are potent pro-inflammatory mediators and may well contribute to the symptoms of HIDS. Since specific leukotriene antagonists have become available and have been found to be safe in children (33), a trial of these agents may be warranted. Alternatively, dietary

measures reducing the generation of cysteinyl leukotrienes, e.g. by adding fish oil(34), might be worthwhile exploring.

Having addressed the pathogenesis of inflammation in HIDS, we then asked why inflammation in this disease is episodic, rather than continuous. As described in **Chapter 7**, it turns out that in HIDS, the mutated MK protein is synthesized even less effectively when temperature is raised. This leads to a worsening of MK deficiency. The activity of the preceding enzyme, HMG-CoA reductase, may be increased to offset the effect of MK deficiency on isoprenoid biosynthesis. However, when MK deficiency is profound (as in Mevalonic aciduria) or drops suddenly (as in HIDS at higher temperatures) MK becomes rate limiting. Raised body temperature is common in response to immunizations, viral infections and strenuous exercise, the usual triggers of fever attacks in HIDS. We therefore hypothesize, that, in vivo, myeloid cells, possibly activated via T-lymphocytes, are exposed to raised body temperature. This would then lead to a progressive MK deficiency, resulting in a shortage of non-sterol isoprenoids, which we think leads to a failure to limit activation of myeloid cells in HIDS. The absence of inflammation between attacks would be explained by the compensatory up regulation of HMG-CoA reductase, resulting in elevated intracellular mevalonate levels. Once, upon an inflammatory trigger, this compensation fails, inflammation, fever and enzyme deficiency spiral out of control.

Figure 4



Current understanding of the pathogenesis of HIDS

In recent years our understanding of the pathogenesis of HIDS has improved considerably (fig.4), but precise mechanisms linking isoprenoid biosynthesis and inflammation have remained elusive. However, it is vital that these be uncovered. HIDS and MA are troublesome, but rare. Autoinflammatory diseases in general are troublesome and not rare. It is in this group of diseases that the control of normal healthy defense mechanisms, i.e. innate immunity and non-specific inflammation, fails. The persistence of such response patterns, which had evolved only to deal with acute microbial threats, becomes deleterious to the host. The devastating effects of chronic inflammation in autoinflammatory disorders are well known and our therapeutic arsenal is often insufficient. The study of "experiments of nature" such as HIDS, should uncover physiologic mechanisms that prevent inflammation becoming chronic. Ultimately, knowledge gained in these rare diseases should lead to better treatment options for both HIDS and the more common autoinflammatory disorders.

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Samenvatting

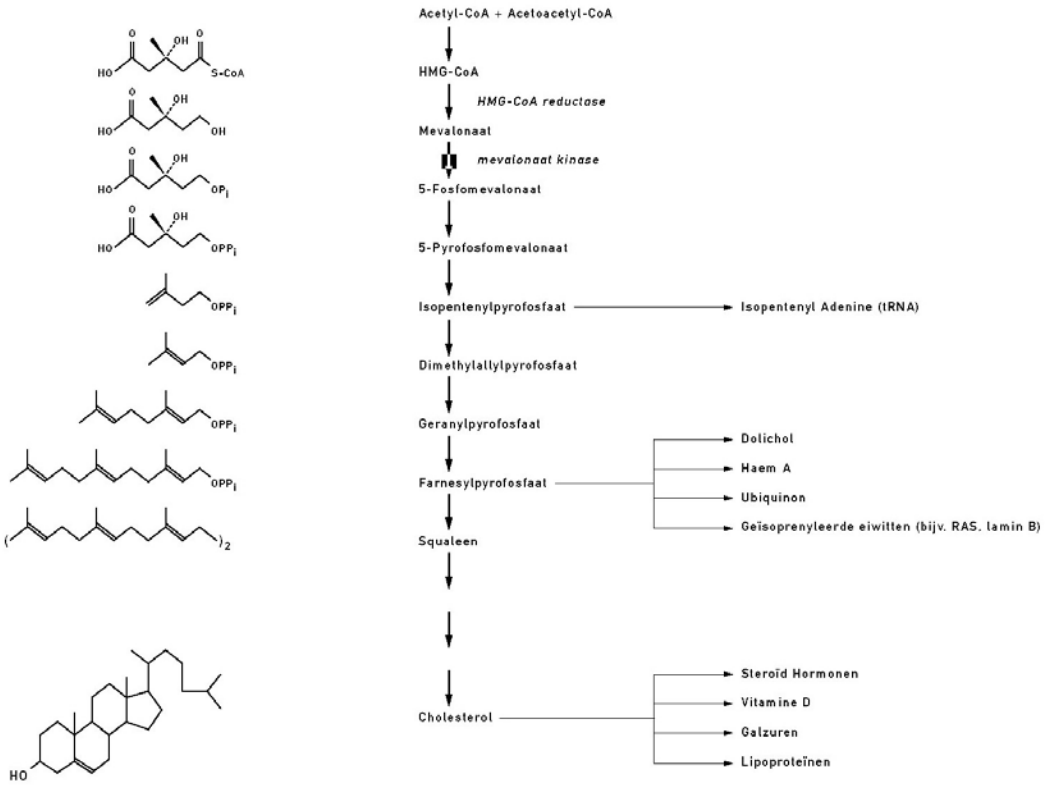
Koorts is meestal het gevolg van een infectieziekte. In zeldzamere gevallen leidt auto-immuniteit, de respons van het afweersysteem op lichaamseigen bestanddelen, tot koorts Er zijn echter patiënten bij wie koorts en andere ontstekingsverschijnselen optreden die niet berusten op infectie of autoimmuniteit. Deze aandoeningen kunnen aangeboren of verworven zijn en staan bekend als autoinflammatoire ziekten. De aangeboren autoinflammatoire ziekten kenmerken zich door recidiverende koortsaanvallen. De bekendste van deze hereditaire periodieke koortssyndromen was de familiale mediterrane koorts (FMF), die voornamelijk voorkomt bij mensen wiens voorouders uit het nabije oosten stammen. Begin jaren 80 van de vorige eeuw (1;2), werd duidelijk dat er in onze streken een niet-mediterraan erfelijk syndroom met periodieke koorts bestond, waarbij het immunoglobuline IgD, een eiwit met onbekende functie, in het serum sterk verhoogd was. Het betrof patiënten die vanaf de kinderleeftijd regelmatig, elke 2 tot 12 weken, een koortsaanval doormaakten die acuut begon, een dag of drie aanhield en gepaard ging met huiduitslag, gewrichtspijnen, braken, buikpijn, diarree en een opvallende lymfeklierzwelling. Sedertdien is onze kennis over dit HyperIgD periodieke koorts syndroom (HIDS) of Dutch type Periodic Fever (DPF) sterk toegenomen. De klinische verschijnselen en het autosomaal recessieve erfelijkheidspatroon waren nauwkeurig in kaart gebracht (3). Daarentegen bleven de drie kernverschijnselen van het syndroom, te weten de verhoging van serum immunoglobulinen A en D, de gegeneraliseerde ontsteking gepaard met hoge koorts en het optreden van ziekteverschijnselen in aanvallen, onbegrepen. Effectieve behandeling was niet voorhanden. Onderzoek naar het

mechanisme van de ontsteking bracht een toegenomen activiteit aan het licht van pro-inflammatoire cytokinen, zoals Interferon (IFN)-γ, Tumor Necrose Factor (TNF)-α, Interleukine (IL)-1β and IL-6 (4-8).

In 1999, werd duidelijk dat tekort aan mevalonaat kinase tot HIDS kon leiden (9;10). Mevalonaat kinase is een vroeg enzym in de isoprenoid biosynthese, de biochemische route waarmee onder andere cholesterol wordt geproduceerd. De ontdekking dat een aangeboren stofwisselingsdefect aan de ontstekingsziekte HIDS ten grondslag lag, riep mogelijk meer vragen op dan zij beantwoordde (11). Wat was nu precies de relatie tussen mevalonaat kinase deficiëntie en HIDS? Wordt HIDS altijd door mevalonaat kinase deficiëntie veroorzaakt? Leidt mevalonaat kinase deficiëntie altijd tot HIDS? En hoe leidt dit stofwisselingsdefect tot gegeneraliseerde ontsteking? Komt dat door ophoping van het substraat (mevalonaat) of een tekort aan een van de vele producten van de isoprenoid biosynthese (fig.1)? In welke cellen of weefsels sorteert deze metabole ontregeling haar inflammatoire effect?.Zijn er, naast wat reeds bekend was, nog meer ontstekingsmechanismen betrokken bij deze ziekte? En hoe kan een persisterend metabool defect tot aanvalsgewijze ziekteverschijnselen leiden?

Deze vragen waren het onderwerp van het beschreven onderzoek

Figuur 1



Schematische weergave van de isopremoïd biosynthese.

Het biochemische pad voert van acetyl-CoA en acetoacetyl-CoA tot een veelheid van eindproducten. Naast cholesterol levert deze route ook non-sterol isoprenoïden als isopentenyl groepen, farnesyl-groepen, dolichol, ubiquinon-10 en haem-A. Het metabole defect bij HIDS is onmiddellijk na HMG-CoA reductase, ter plaatse van mevalonaat kinase.

In **hoofdstuk 2**, beschrijven wij een groep kinderen met HIDS (12). Het bleek dat niet alle HIDS patiënten aan mevalonaat kinase deficiëntie leden. Ongeveer 80% van de onderzochte patiënten hadden volledig normale activiteit van het enzym. Inmiddels is deze bevinding ook bij volwassen patiënten bevestigd (13). De klinische kenmerken maken geen onderscheid mogelijk tussen HIDS met en HIDS zonder mevalonaat

kinase deficiëntie Desondanks komen sommige verschijnselen, zoals begin van de ziekte op de zuigelingenleeftijd, huiduitslag en diarree, meer voor bij de patiënten met mevalonaat kinase deficiëntie. Deze groep wordt aangeduid als HIDS in engere zin of DPF. Een verhoogd serum IgD is klaarblijkelijk niet specifiek voor mevalonaat kinase deficiëntie. Integendeel, bij alle hereditaire periodieke koorts syndromen en ook bij niet-erfelijke autoinflammatoire ziektebeelden als het. Periodieke koorts, adenitis, faryngitis, stomatitis aftosa (PFAPA) syndroom (14) zijn patiënten beschreven met een verhoogd serum IgD(15;16). Het is dan ook niet verwonderlijk dat het serum IgD verhoogd kan zijn bij patiënten met periodieke koorts bij wie vooralsnog geen syndroomdiagnose mogelijk is. Het opvallende aan HIDS in engere zin, is echter dat het serum IgD veel vaker verhoogd is dan bij andere ziektebeelden. Het is niet bekend hoe bij mevalonaat kinase deficiëntie een stijging van het serum IgD wordt veroorzaakt. Uiteindelijk zal inzicht in het onderliggende mechanisme licht kunnen doen schijnen op het verband tussen isoprenoïd biosynthese en het immuunsysteem. Het serum IgD is overigens niet bij alle mevalonaat kinase deficiënte patiënten verhoogd (17) (9). Daarmee ontstaat een semantisch probleem. Verschillende pathologische entiteiten worden met één term (HIDS) aangeduid. Omgekeerd worden de uiteenlopende uitingsvormen van mevalonaat kinase deficiëntie juist verschillend benoemd.

Dat laatste wordt belicht in **hoofdstuk 3.** Immers, naast de patiënten met periodieke koorts en gegeneraliseerde ontstekingsverschijnselen, al of niet met een hoog serum IgD, zijn er die tevens pre- en postnatale groeivertraging hebben, achterblijven in neurologische ontwikkeling en gelaatsmisvormingen vertonen. Dit laatste fenotype staat bekend als mevalon acidurie (MA) (18). Wij konden aantonen dat er unieke mutaties voorkwamen bij HIDS die niet bij mevalon acidurie patiënten werden

aangetroffen (19). All HIDS patiënten hadden een *MVK*-gen met de mutatie 1129 G>A die codeert voor de aminozuur substitutie V377I. Bij MA patiënten kwam deze mutatie niet voor. Het eiwit met de V377I verandering geeft een minder diepe mevalonaat kinase deficiëntie dan de eiwitveranderingen die werden aangetroffen bij mevalon acidurie patiënten. Klaarblijkelijk is het deze relatief milde mevalonaat kinase deficiëntie die tot het HIDS fenotype leidt. Overigens zijn HIDS en MA waarschijnlijk uitersten van een klinisch continuüm. Zo zijn er patiënten met HIDS op basis van milde enzymdeficiëntie die desondanks wel degelijk mentaal geretardeerd zijn. Het viel op dat slechts enkele HIDS patiënten homozygoot waren voor de V377I mutatie. Waarschijnlijk ontsnappen de meeste van hen aan de aandacht van medici en het is aannemelijk dat vele van hen geheel klachtenvrij zijn.
Mevalonaat kinase deficiëntie leidt zowel tot een ophoping van het substraat, mevalonzuur, als tot een verminderde productie van isoprenoïden (fig.1).

Welke van deze twee veranderingen leidt tot ontsteking werd onderzocht in **hoofdstuk 4**. In een ex-vivo model, bepaalden wij de IL-1β secretie van normale geïsoleerde mononucleaire witte bloedcellen na stimulatie via de aanwezige T-lymfocyten. In dit model leidde reductie van de isoprenoïd productie tot en toegenomen IL-1β secretie. Blootstelling aan mevalonaat leidde daarentegen niet tot verhoogde IL-1 β secretie. Cellen van HIDS patiënten lieten juist een vermindering van de IL-1β secretie zien in aanwezigheid van mevalonaat. Een soortgelijke vermindering in IL-1β secretie namen wij waar wanneer de blokkade in isoprenoïd biosynthese werd omzeild. Dat kon door aan de cellen metabolieten toe te voegen die stroomafwaarts van de belemmering in de biochemische route kunnen instromen. De beschikbaarheid van mevalonaat, dat stroomopwaarts van de belemmering bij HIDS

het pad instroomt, bleek onverwacht van cruciaal belang voor de cellen van HIDS. De IL-1β secretie door deze cellen nam niet alleen af bij toevoeging van mevalonaat, zij nam ook toe, wanneer de mevalonaat productie werd onderdrukt door lovastatine. Deze waarneming geeft aan dat de behandeling van mevalonaat kinase deficiëntie zich zou moeten richten op het herstel van isoprenoïd productie in plaats van op de reductie van mealonaat productie.Het blijft op dit moment onduidelijk aan welke van de vele producten van de isoprenoïd biosynthese een dusdanig tekort ontstaat dat ontstekingsverschijnselen optreden. A fortiori is het onduidelijk hoe een dergelijk tekort tot ontsteking leidt.

Evenmin was duidelijk waar, dat wil zeggen in welk weefsel of celtype, belemmering van de isoprenoïd biosynthese tot ontsteking leidde.

In **hoofdstuk 5** onderzochten wij daarom leukocyten van HIDS patiënten tijdens koorts aanvallen en in aanvalsvrije episoden. Het was van verscheidene leukocyten subpopulaties denkbaar dat zij geactiveerd zouden zijn bij deze aandoening. Activatie van B-lymfocyten kon worden verwacht op grond van de polyclonale verhoging van serum IgD en IgA bij HIDS (20). Betrokkenheid van T-lymfocyten, was reeds vermoed vanwege de hoge serum concentratie van interferon-gamma tijdens koortsaanvallen(6). Mononucleaire fagocyten konden worden verondersteld geactiveerd te zijn. Immers, verhoogde productie van ontstekingsmediatoren afkomstig van deze celpoplatie waren zowel in- als ex-vivo waargenomen bij HIDS patiënten (7;8). Ook de verhoogde excretie van neopterine en Leukotrïeen-E4 in de urine wees op activatie van mononucleaire fagocyten (8)(hoofdstuk 6). Betrokkenheid van neutrofiele granulocyten kon worden vermoed, aangezien granulocytose tijdens de koortsaanvallen van HIDS regel is (3). Hoewel er discrete aanwijzingen voor T-cel activatie waren, vielen vooral de veraderingen onder de myeloïde cellen op. Het

aantal neutrofiele granulocyten was verviervoudigd, het aantal monocyten verdrievoudigd. Bovendien waren deze cellen geactiveerd, zoals bleek uit de verhoogde expressie van het oppervlakte-molecuul CD64. Hiermee is nog niet aangetoond dat myeloïde cellen als eerste geactiveerd worden bij de ontstekingsepisoden van HIDS. Mogelijk worden deze leukocyten indirect geactiveerd door een andere celpopulatie, zoals T-cellen of niet-circulerende cellen of weefsels. Echter, een centrale rol voor granulocyten en monocyten zou overeen komen met hetgeen bij de andere erfelijke periodieke koorts syndromen is gevonden.
In **hoofdstuk 6** onderzochten wij of cysteinyl leukotriënen, ontstekingsmediatoren betrokken bij de koortsaanvallen van mevalon acidurie, ook een rol speelden bij het mildere fenotype van mevalonaat kinase deficiëntie, HIDS. Uit de verhoogde concentratie van leukotrïeen-E4 (LTE_4)in de urine kon worden geconcludeerd dat dit tijdens koortsepisoden het geval was. Welke cellen of weefsels deze mediatoren produceerden werd niet onderzocht, maar, gezien de bevindingen in hoofdstuk 4, is het plausibel dat mononucleaire fagocyten hiervoor verantwoordelijk zijn. Cysteinyl leukotriënen zijn krachtige pro-inflammatoire mediatoren, die waarschijnlijk substantieel bijdragen aan de klinische verschijnselen van HIDS-patiënten. Aangezien er tegenwoordig veilige en effectieve leukotrïeen antagoniten beschikbaar zijn, lijkt een studie naar het effect van dergelijke middelen bij HIDS zinvol.
Ten slotte onderzochten wij, waardoor de onstekingsverschijnselen bij deze aandoening periodiek in plaats van continu zijn. Het blijkt, zoals beschreven in **hoofdstuk 7**, dat het mutante mevalonaat kinase eiwit van HIDS patiënten niet effectief kan worden aangemaakt wanneer de temperatuur toeneemt. Het resultaat is een verergering van de mevalonaat kinase deficiëntie. Dit werd zowel in-vitro als in-vivo waargenomen. De activiteit van het voorafgaande enzym, HMG-CoA reductase,

kan toenemen om te compenseren voor het effect van mevalonaat kinase deficiëntie op de isoprenoïd biosynthese. Echter, bij diepe mevalonaat kinase deficiëntie (zoals bij patiënten met mevalon acidurie) of bij een plotselinge afname van de mevalonaat kinase-activiteit (zoals bij HIDS, wanneer de temperatuur oploopt), wordt mevalonaat kinase de flessenhals van deze biosynthetische route. Verhoogde lichaamstemperatuur kan het gevolg zijn van infecties, inentingen of zware lichamelijke inspanning, omstandigheden waarvan bekend is dat zij vaak aanvallen bij HIDS-patiënten uitlokken.

Gezien het voorgaande veronderstellen wij, dat myeloïde cellen, al dan niet geactiveerd via T-cellen, in vivo bloot worden gesteld aan een verhoogde lichaamstemperatuur. Dit resulteert in een progressieve mevalonaat kinase deficiëntie, waardoor een tekort ontstaat aan bepaalde isoprenoïden. Dit tekort veroorzaakt op haar beurt een ongecontroleeerde activatie van myeloïde cellen. Het ontbreken van ontstekingsverschijnselen tussen de aanvallen zou mogelijk kunnen worden verklaard door een compensatoire verhoging van de HMG-CoA reductase activiteit. Wanneer deze compensatie, als gevolg van een inflammatoire stimulus, tekort schiet, zou een vicieuze cyclus kunnen ontstaan van koorts, enzym deficiëntie en ontsteking.

Hoewel onze kennis van de pathogenese van HIDS in de afgelopen jaren belangrijk is toegenomen, kennen wij het precieze mechanismen die gestoorde isoprenoïd biosynthese met ontsteking verbindt nog niet. Het is echter essentieel dat deze worden opgehelderd. Alleen op die manier kan een effectieve behandeling voor deze andening worden gevonden. Nu is die nog niet oorhanden.

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After secondary education at the Barlaeus Gymnasium in Amsterdam, he entered medical school at the University of Amsterdam in 1976. In 1980-81, as an ITT-international exchange student, he studied the biochemistry of osteocalcin at the laboratory of Prof Paul Galop at Harvard Medical School, Boston. After resuming medical school, he graduated with honors in 1986.

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Mutations in the gene encoding mevalonate kinase cause hyperimmunoglobulinemia D and periodic fever syndrome

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Hyperimmunoglobulinemia D and periodic fever syndrome (HIDS; MIM 260920) is an autosomal recessive disorder characterized by recurrent episodes of fever associated with lymphadenopathy, arthralgia, gastrointestinal dismay and skin rash (1;2). Diagnostic hallmark of HIDS is a constitutively elevated level of serum immunoglobulin D (IgD) although patients have been reported with normal IgD levels(2). In search for the underlying defect in HIDS, we analyzed urine of several patients and discovered increased concentrations of mevalonic acid during severe episodes of fever but not in between crises. Subsequent analysis of cells from four unrelated HIDS patients revealed strongly reduced activities of mevalonate kinase (MK), a key enzyme of isoprenoid biosynthesis. Sequence analysis of the patients' MK cDNAs identified three different mutations of which one was common to all four patients. Expression of the mutant cDNAs in *Escherichia coli* showed that all three mutations affect the activity of the encoded proteins. Moreover, immunoblot analysis demonstrated a deficiency of MK protein in patients' fibroblasts indicating a protein destabilizing effect of the mutations. The identification of an enzyme defect in isoprenoid biosynthesis in patients with periodic fever and dysregulation of their immune system creates new challenges for the elucidation of the pathogenesis of periodic fever.

On performing organic acid analysis of urine and, subsequently, plasma from an 18-year old patient obtained during a severe episode of fever we noticed significantly increased concentrations of mevalonic acid (patient 1; Table 1 and 2). Because this patient had suffered from recurrent episodes of fever associated with clinical signs characteristic of HIDS since the age of 16 months (although he never displayed elevated serum IgD levels), we examined two additional patients clinically diagnosed with HIDS (patients 2 and 3; Table 1). These patients also showed increased concentrations of mevalonic acid in their urine during but not in between episodes of fever (Table 2).

Table 1 • Clinical features of the four HIDS patients and clinical features characteristic for HIDS and mevalonic aciduria[a]

Clinical features	Patient number				HIDS[b]	Mevalonic aciduria[c]
	1	2	3	4		
Recurrent fevers	+	+	+	+	+	+
Lymphadenopathy	+	+	+	+	+	+
Skin lesions/rash	+	+	+	+	+	+
Abdominal pain/diarrhea	+	+	+	+	+	+
Arthralgia	+	+	+	+	+	+
Psychomotor retardation	–	+	–	–	–	+/+++
Failure to thrive	–	–	–	–	–	+/+++
Hypotonia/myopathy	+	–	–	–	–	–/++
Ataxia/cerebellar atrophy	–	–	–	–	–	–/+++
Hepatomegaly	+	–	–	+	–	–/+
Cataracts	–	–	–	–	–	–/+
Dysmorphic features	–	–	–	–	–	–/+
Age of onset (months)	16	3	7	15		
Serum IgD (U/ml) Min.	12	960	222	47	>100	?
Max.	32	4244	350	192		

[a], symbols used: +, present; ++, moderate; +++, severe; –, absent; ?, unknown;

[b], adapted from ref. 2; [c], adapted from ref. 6.

The increased concentrations of mevalonic acid suggested a defect in the metabolism of mevalonate, which is the physiological substrate of mevalonate kinase (MK). This dimeric protein, reported to be localized in both cytosol and peroxisomes (3), is a key enzyme of isoprenoid biosynthesis and catalizes the phosphorylation of mevalonate to produce 5-phosphomevalonate (4). When we measured the activity of MK in lymphocytes of four HIDS patients we found markedly reduced activities ranging from 1.3 to 3.2% of the mean activity measured in control lymphocytes (Table 2). Analysis of lymphocytes of the parents of patients 1, 2 and 3 showed intermediate MK activities ranging from 22 to 46% of the mean activity in control lymphocytes and indicating heterozygosity for the defect which is in accordance with the presumed autosomal recessive inheritance of HIDS.

Table 2 • Biochemical data and mutations of HIDS patients and their relatives

Individual	Urinary Mevalonic acid[a]	MK activity in lymphocytes[b]	Mutation	Coding effect
Control subjects	0.08-0.3	95.8±17.0	–	–
HIDS patient 1	8.3-9.6	2.0 (2.1%)	59A>C	H20P
			1129G>A	V377I
Mother	ND	21.1 (22%)	59A>C	H20P
Father	ND	44.0 (46%)	1129G>A	V377I
Sister	ND	30.5 (32%)	1129G>A	V377I
Brother	ND	117 (122%)	no mutation	–
HIDS patient 2	6.4	1.3 (1.2%)	59A>C	H20P
			1129G>A	V377I
Mother	ND	24.1 (25%)	ND	–
Father	ND	38.7 (41%)	ND	–
HIDS patient 3	5.3-27.8	1.9 (2%)	803T>C	I268T
			1129G>A	V377I
Mother	ND	31.0 (33%)	ND	–
Father	ND	25.4 (27%)	ND	–
HIDS patient 4	ND	3.3 (3.4%)	1129G>A	V377I

[a], mmol/mol creatinine; [b], pmol/min/mg protein; ND, not determined.

The combination of a deficient MK activity and increased concentrations of excreted mevalonic acid, but then constitutive and much more massive, are also characteristic for mevalonic aciduria (MIM 251170), a rare and severe, progressive, multi-systemic disorder associated with periodic fever and caused by mutations in the gene encoding MK (5-8). To determine the relationship between HIDS and mevalonic aciduria, we measured the MK activities in cultured skin fibroblasts from HIDS patients 1, 2 and 3 and two patients with mevalonic aciduria. Virtually no MK activity was detected in the cells of both mevalonic aciduria patients (0.2% residual activity) while the activities in the cells of the HIDS patients ranged from 0.8 to 3.6% of the mean activity measured in control fibroblasts.

Sequence analysis of RT-PCR amplified MK cDNAs identified the same 1129G>A mutation in all four HIDS patients (Table 2). Patients 1 to 3 were compound heterozygous for this mutation and an additional missense mutation, while patient 4 appeared homozygous for the 1129G>A mutation. However, it can not be excluded at present that this latter patient is compound heterozygous for this mutation and a second mutation that produces an unstable mRNA. The 1129G>A mutation changes the valine at position 377 of the MK amino acid sequence (7) into an isoleucine (V377I). Comparison of the human MK amino acid sequence to the sequences of rat and mouse MK (82% and 81% identity with human MK, respectively) revealed that this valine is fully conserved (not shown). Nevertheless, to exclude that this relatively mild conversion was due to a common polymorphism, we analyzed MK cDNAs of 24 control subjects, including 15 subjects of Dutch origin, for the presence of this V377I allele and did not detect any. The two additional missense mutations identified in patients 1 and 2 (59A>C) and in patient 3 (803T>C) change the histidine at position 20 into a proline (H20P) and the isoleucine at position 268 into a threonine (I268T), respectively. All three mutations differ from the two previously reported mutations causing mevalonic aciduria

(N301T and A334T) (7;8). The autosomal recessive nature of the disorder was confirmed by the heterozygous presence of one of the two mutations of patient 1 in the MK cDNAs of his symptom-free parents and sister (Table 2) and fits with the reported location of the MK gene on chromosome 12 (Ref. 7).

The identification of the same V377I allele in the four unrelated HIDS patients strongly suggested that this allele is responsible for the presentation of HIDS. Furthermore, the residual MK activity measured in cells of these patients suggested that the V377I allele produces a mutant MK protein with either a strongly reduced activity or stability or both. Therefore, we first examined the effect of the different mutations on the MK activity by means of expression of the wild type and the three mutant proteins as a fusion to glutathione S-transferase (GST) in *Escherichia coli*. Previously, it had been shown that this fusion stabilizes the MK enzyme and hardly changes its kinetic properties when compared to MK purified from liver(8). An additional advantage of this approach is that the MK activities can be normalized for the activities of GST to allow direct comparison between the wild type and mutant enzymes. The enzyme measurements in *E. coli* lysates showed that both the H20P and the I268T mutation have a dramatic effect on the activity of the respective MK proteins (Fig. 1).

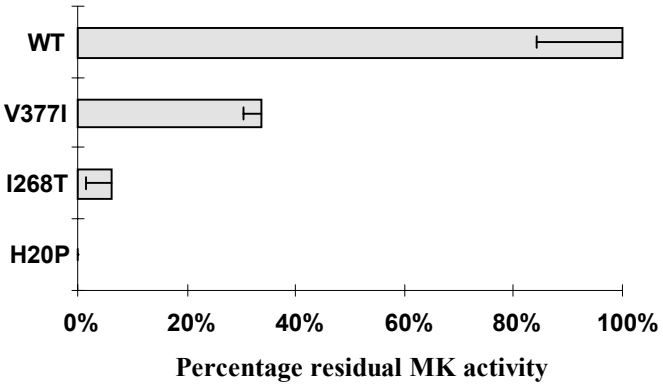

Fig. 1 Expression of MK cDNAs in *Escherichia coli*. The coding sequences of wild type and mutant MK cDNAs were amplified by RT-PCR and expressed as a fusion with glutathione S-transferase (GST) in *E. coli*. The MK enzyme activities were measured in *E. coli* lysates and normalized for GST enzyme activities to correct for differences in expression. MK/GST activity ratios of the mutant fusion proteins are presented as the percentage of the mean ratio of the wild type fusion protein. The results are the mean of four independent measurements.

The V377I mutation also causes a significant decrease in the MK activity, but the relative residual activity was still approximately ten fold higher than the activity measured in the cells of the HIDS patients. However, when we performed immunoblot analysis of fibroblast lysates of the patients, we hardly detected any MK protein while in lysates of control fibroblasts the protein is readily visible (Fig. 2). Together, these results indicate that although the V377I mutation has some effect on the enzyme activity, it predominantly affects the stability of the MK protein.

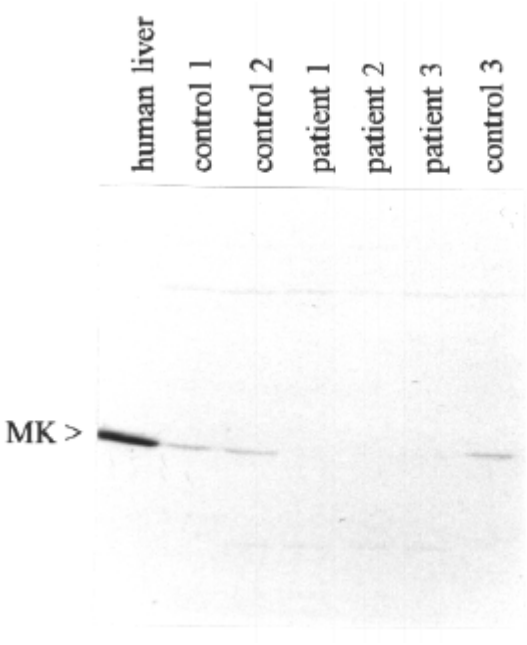

Fig. 2 Immunoblot analysis of fibroblast lysates of HIDS patients. Cell lysates of cultured skin fibroblasts of three control subjects and HIDS patients 1, 2 and 3 were resolved by SDS-PAGE (100 μg of total protein per lane), blotted onto nitrocellulose and analyzed with a polyclonal antibody against human MK. For reference, a liver cell lysate from a control subject (40 μg of total protein) is included.

In conclusion, our results indicate that HIDS is the consequence of a strongly reduced but not completely deficient activity of mevalonate kinase and therefore could be considered as a mild presentation of mevalonic aciduria. This also is apparent when the clinical features associated with HIDS are compared to those associated with mevalonic aciduria (Table 1). The fact that one of the HIDS patients did not have an elevated level of serum IgD indicates that this elevation, which is often associated with HIDS, is an epiphenomenon.

The unexpected discovery of a metabolic defect in the isoprenoid biosynthesis in patients with periodic fever and a dysregulation of their immune system creates new challenges for the understanding and elucidation of the processes underlying periodic fever or fever in general. Isoprenoids are important intermediates in the generation of several classes of compounds, including sterols, dolichol, ubiquinone and prenylated proteins, which can be involved in a variety of cellular processes such as cell growth and differentation, glycosylation, signal transduction and electron transport (4). At this moment, it is unclear which of these processes are linked to the pathogenesis of HIDS or to other syndromes associated with periodic fever such as systemic juvenile rheumatoid arthritis, familial Mediterranean fever and familial Hibernian fever (9).

Methods

Patients. All patients used in this study were unrelated to each other and of Dutch origin except for patient 3 who had a father of Indonesian origin. Patients 1-3 were male and patient 4 female. The diagnosis of HIDS was based on clinical signs and, except for patient 1, elevated serum IgD levels (Table 1).

Mevalonic acid measurements. Concentrations of mevalonic acid in urine and plasma were determined by stable isotope dilution gas chromatography - mass spectrometry of the trimethylsilyl ether esters using $^{2}H_{7}$-mevalonolactone (C/D/N Isotope Inc) as internal standard (10).

Enzyme assays. MK was measured using ^{14}C-labeled mevalonate as the substrate (11). Gluthione-S-transferase activity was assayed on a spectrophotometer at 335 nm by measuring the formation of the conjugate of glutathione and 1-chloro-2,4-dinitrobenzene (12)

Mutation analysis of human MK cDNA. First strand cDNA was prepared from total RNA isolated from lymphocytes or fibroblasts as described (13). Two sets of MK-specific primers with -21M13 or M13rev extensions were used to amplify MK cDNA in two overlapping fragments by RT-PCR. The PCR fragments were sequenced in both directions by means of -21M13 and M13rev fluorescent primers on an ABI 377A automated DNA sequencer according to the manufacturer's protocol (Perkin-Elmer).

Expression of mevalonate kinase cDNAs in *E. coli*. The coding sequences of wild type and mutant MK cDNAs were amplified by PCR, cloned in frame with the coding sequences of gluthathione-S-transferase in pGEX-4T (Pharmacia) and sequenced to exclude Taq

polymerase introduced errors. *E. coli* DH5α cells were transformed with the resulting expression plasmids, induced for 2 hours with 2 mM IPTG and subsequently lysed by sonication.

Antibody preparation and immunoblot analysis. A synthetic peptide corresponding to amino acid residues 76 to 90 of human mevalonate kinase[7] was conjugated with keyhole limpet hemocyanin and used to produce antibodies in a rabbit (Eurogentech). The crude antiserum was affinity-purified on a column containing MK-GST coupled to cyanogen bromide sepharose (Pharmacia). For immunoblot analysis, the affinity-purified antibodies were used at a 1:100 dilution and antigen-antibody complexes were visualized using anti-IgG-alkaline phosphatase conjugate.

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