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# A boy with X-linked hyper-IgM syndrome and natural killer cell deficiency

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**SUMMARY**

We present a boy with hyper-IgM syndrome with a previously not reported mutation in the CD40 ligand gene. He also had a concomitant natural killer (NK) cell deficiency. He had no $CD56^+$ or $CD16^+$ cells and no NK activity as determined in 4 h chromium release cytotoxicity assay. After 5 days in culture with IL-2-containing medium, however, his peripheral blood mononuclear cells lysed both NK-sensitive and NK-resistant targets, showing that he had lymphokine-activated killer cell precursors in the circulation. Due to the associated neutropenia, he was treated with granulocyte colony-stimulating factor (G-CSF) and responded well. In the same period we observed a transient increase in the number of NK cells. Isolated NK cell deficiencies are extremely rare. We suggest that the defect in our patient is part of the hyper-IgM syndrome, probably representing the phenotype of the new mutation described. Thus, it is possible that both the neutropenia and the NK cell deficiency are due to lack of growth-promoting signals normally delivered by the CD40 ligand.

**Keywords** Immunodeficiency CD40 ligand cytotoxicity G-CSF LAK cells

## INTRODUCTION

Natural killer (NK) cells constitute 10–19% of the mononuclear cell population in peripheral blood in adults, and slightly less (8–17%) during childhood [1]. These cells appear to represent a first-line defence against a wide variety of malignant or virally transformed cells, capable of spontaneous cytotoxic activity [2, 3]. NK cells express the low-affinity receptor for the Fc portion of IgG (CD16), important for antibody-dependent cellular cytotoxicity (ADCC). They also express the neural cell adhesion molecule (N-CAM) homologue, CD56 [2,4]. This epitope is commonly used as a marker for NK cells, although certain T cell subsets [5] and even non-lymphoid cells may also express this molecule [6].

Since T cells and NK cells are remarkably similar with respect to the expression of different membrane markers and immune effector functions, a common progenitor distinct from the toti-potent lymphoid progenitor is anticipated for these two cell lineages [7,8]. However, in contrast to cytotoxic T cells, NK cell effector functions are not restricted by self MHC molecules, and previous sensitization is not needed [7]. For both cell types the activity can be greatly enhanced by IL-2. These lymphokine-activated killer (LAK) cells can lyse a broad range of NK-resistant tumour targets in a non-MHC-restricted manner [9].

NK cell deficiencies have been described as part of different clinical syndromes such as the Chediak–Higashi syndrome, X-linked lymphoproliferative disorders, leucocyte adhesion deficiency, severe combined immunodeficiency, AIDS and lymphomas [2,10]. These syndromes are generally not associated with a total lack of NK cells. Isolated NK cell deficiencies are extremely rare, and this has made it difficult to demonstrate the importance of NK cell activity in man [3]. However, Biron *et al.* described a young woman with life-threatening herpes virus infections and a total lack of NK cells [11]. In this patient, no LAK cell activity could be induced after *in vitro* treatment with IL-2, suggesting that the LAK phenomenon is primarily dependent on NK cell precursors.

We present here a patient with apparent lack of NK cells and NK function, but with inducible LAK activity. Our patient suffers from a hyper-IgM syndrome, which is usually characterized by severe opportunistic infections (typically *Pneumocystis carinii*) and low or undetectable serum levels of IgG, IgA and IgE, while the level of IgM is normal or elevated. There is commonly an associated neutropenia. Autoimmune haemolytic anaemia and thrombocytopenia are also reported, but NK cell deficiency has not been described as part of the syndrome [12].

Recently, the molecular basis for the hyper-IgM syndrome has been defined as mutations in the gene encoding the CD40 ligand (CD40L), which is expressed on activated T cells [13]. Binding of this molecule to its receptor (CD40) expressed on B cells is of critical importance for immunoglobulin heavy-chain class switching and rescue of B cells from apoptosis in the germinal centres [14].

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## CASE REPORT

This boy was the first child of healthy, non-consanguinous Norwegian parents. In the neonatal period he showed poor weight gain, but he had no serious infections until at 8 months old he was hospitalized with a severe interstitial pneumonia necessitating ventilator treatment. *Pneumocystis carinii* was found in tracheal secretions by immunofluorescence and toluidin blue staining. Immunological investigations showed the normal total numbers of T and B cells, while NK cells, as determined by $CD56^+$ expression, were not detected. Mitogen responses were normal. Quantification of immunoglobulins revealed undetectable IgG (<0·04 g/*l*), IgM was 1·1 g/*l* and IgA was barely detectable (0·1 g/*l*). The patient fully recovered after treatment with high-dose trimethoprim-sulphamethoxazol and immunoglobulin (Sandoglobulin) intravenously. During the first days of immunoglobulin replacement therapy he experienced a short-lived increase in the IgM level to 10·3 g/*l* after 10 days, and a subsequent decrease to 1·9 g/*l* after the next 30 days. Subsequently, IgM levels were in the upper normal range. Due to an increased frequency of upper respiratory tract infections, he was re-evaluated when he was 4 years old. The T and B lymphocyte counts were still normal, while the number of NK cells was profoundly diminished. In addition, he had developed a neutropenia with an absolute neutrophil count (ANC) of $0{\cdot}14 \times 10^9/l$. His bone marrow was hypocellular with a decreased myelopoiesis and a possible maturation block between promyelocytes and myelocytes. No anti-neutrophil antibodies were detected. Accordingly, he was started on granulocyte colony-stimulating factor (G-CSF; Neupogen) treatment, and his ANC reached $1{\cdot}5 \times 10^9/l$ at a dose of 5 $\mu$g/kg per day subcutaneously. There was a prompt clinical effect, with no more serious infections. In addition to the G-CSF therapy, which he has now received for 3 years, he is now treated with subcutaneous immunoglobulin and prophylactic doses of trimethoprim-sulphamethoxazol.

## MATERIALS AND METHODS

*Isolation of cells*

For functional testing, 10 ml heparinized peripheral blood were collected, and mononuclear cells were separated by density gradient centrifugation (Lymphoprep, Nycomed, Norway). The isolated cells were resuspended in medium (RPMI 1640) supplemented with 1 mM sodium pyruvate, non-essential amino acids, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 10% fetal calf serum (complete medium). To generate LAK cells, mononuclear cells were cultured in complete medium supplemented with human recombinant IL-2 100 U/ml (Cetus Corp., Emeryville, CA). Peripheral blood mononuclear cells (PMBC) were also collected from three other patients with hyper-IgM syndrome and from healthy blood donors.

*Mutation analysis*

Genomic DNA from the patient was amplified by polymerase chain reaction (PCR) using intronic primers for CD40L gene.

Single-strain conformation polymorphism (SSCP) analysis was performed loading the amplification products onto 0·5 × MDE gel plus 5% glycerol, running at 4°C for 20 h and then staining with silver nitrate. PCR amplification products were purified and direct sequenced using cyclist kit (Stratagene) using the same primers used for the amplification.

The primers used for the amplification of Exon III were: A: 5′ AAATGACCTCTTGCATGCTTC 3′; B: 5′ TCATAGAAATTAGCAAATAG 3′.

*Phenotypic analysis*

Freshly isolated or cultured mononuclear cells were resuspended in cold Hanks' balanced salt solution (HBSS) with 0·02% sodium azide in V-bottomed plates in 50-$\mu$l volumes, and 2·5 $\mu$l fluorescein-conjugated MoAb was added for 30 min on ice. The antibodies used were UCHT1 (CD3), MT310 (CD4), DK25 (CD8), MHM23 (LFA1), all from Dakopatts (Glostrup, Denmark), NKH1 (CD56) and 2H4 (CD45RA) from Coulter Immunology (Hialeah, FL), Leu-11c (CD16) from Beckton Dickinson (Mountain View, CA), TCR$\delta$1 (T cell $\gamma\delta$-receptor) from T Cell Science (Cambridge, MA), OKT-11 (LFA2) from ATCC (Rockville, MD) and 3D5 (VLA4), a gift from Dr Gustav Gaudernack. (The Norwegian Radium Hospital, Oslo, Norway).

Two-colour immunofluorescene analysis was performed using a FACScan flow cytometer (Beckton Dickinson) and LYSYS II software.

*Cytotoxicity assays*

A standard 4-h chromium release assay was performed, as described [15]. Briefly, target cells were labelled with 10 MBq $^{51}$Cr-sodium-chromate in 0·3 ml medium for 1–2 h at 37°C. After extensive washing, the cells were resuspended in complete medium and 5000 cells were added per well in round-bottomed microtitre plates. As target cells we used K562 (a NK-sensitive erythroleukaemic cell line) and Daudi (a lymphoma cell line resistant to NK cells, but susceptible to LAK cell cytotoxicity).

Effector cell lymphocytes, either freshly isolated or LAK cells, were added in appropriate concentrations to obtain effector/target cell ratios (E/T) ranging from 40/1 to 5/1. Maximal lysis was achieved by incubating target cells with 5% Triton X-100, and spontaneous release by incubation with medium alone. The plates were harvested with the Skatron supernatant collection system (Skatron, Lier, Norway). All cultures were performed in triplicates. Cytotoxicity was calculated as:

$$\text{Percent specific lysis} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximal release} - \text{spontaneous release})} \times 100$$

T cell cytotoxicity was tested in a redirected system utilizing anti-human CD3 MoAbs in the membrane of the target cells, as described by Spits *et al.* [16].

*Blocking studies*

To investigate the possible involvement of various adhesion molecules in the cytolytic process, we also tested the cytotoxicity after preincubating the effector cells for 30 min with saturating amounts of MoAbs against CD2, CD11a, CD18 or CD49d. The 5B5 anti-fibroblast antibody (IgG1) served as control. Effector/target ratios in these experiments were 20/1.

## RESULTS

*Genotype*

Amplification of genomic DNA using oligonucleotide intronic primers that flank intron/exon boundaries in the CD40L gene, followed by sequencing, revealed that the patient carries a T to G substitution at nt + 1, in the invariant gt donor splice site of

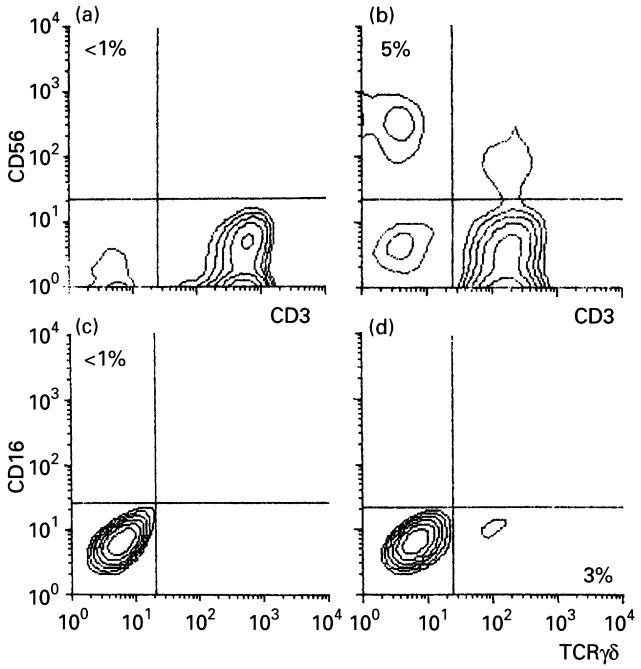


**Fig. 1.** Phenotype of the patient's mononuclear cells. Two-colour FACS analysis of freshly isolated mononuclear cells, showing the absence of $CD56^+$ natural killer (NK) cells (a). No selective expansion of other subsets known to exhibit non-MHC-restricted cytotoxicity was noted after IL-2 stimulation (c,d). After 1 year of treatment with granulocyte colony-stimulating factor (G-CSF), the patient had NK cells, and $CD3^+CD56^+$ (double-positive) T cells were also noted after 5 days culture with IL-2 (b).

intron 3. This mutation has not previously been reported in patients with a CD40L defect.

*Phenotype*

The phenotype of the freshly isolated mononuclear cells from this patient was predominantly $CD3^+CD56^-$ T cells. There were no detectable NK cells ($CD3^-CD56^+$) (Fig. 1a). A dominance of $CD4^+$ (T helper cells) and $CD45RA^+$ cells (a marker associated with naive lymphocytes) was noted (data not shown), but both were within the normal range [1]. The common adhesion molecules (LFA-1, LFA-2, intercellular adhesion molecule-1 (ICAM-1) and very late antigen-4 (VLA-4)) were fully expressed compared with normal controls (data not shown). This phenotypic profile did not change substantially after culture with IL-2 100 U/ml for 5 days. No selective expansion of any subpopulation known to exert non-MHC-restricted cytotoxic activity ($CD16^+$ or $TCR\gamma\delta^+$ T cells) was seen (Fig. 1c,d), and no T cells acquired the $CD3^+CD56^+$ (double-positive) phenotype.

The phenotypic profile has been repeatedly studied, and it remained more or less unchanged until October 1992, when we observed an increase in the number of NK cells, although still below the normal range (Fig. 2). Interestingly, this occurred after starting the G-CSF treatment. However, the most recent values show a decreasing tendency. $CD3^+CD56^+$ (double-positive) T cells were also observed, especially after culture with IL-2 (Fig. 1b).

By contrast, the other three patients with hyper-IgM syndrome had NK cell numbers within the normal range (data not shown).

*Cytotoxicity*

Freshly isolated peripheral blood lymphocytes from the patient

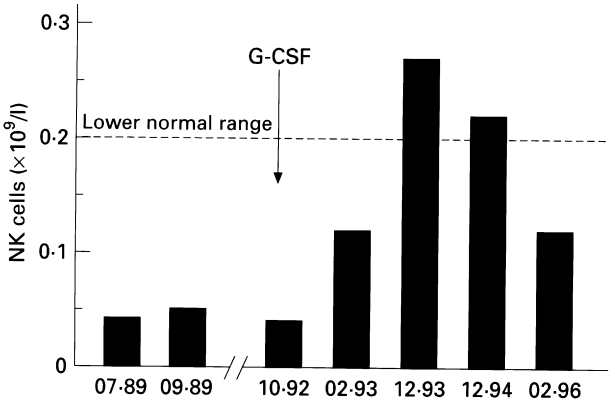


**Fig. 2.** The number of natural killer (NK) cells increased slightly after granulocyte colony-stimulating factor (G-CSF) treatment. The number of NK cells was calculated as the $CD56^+$ fraction (% of all mononuclear cells) multiplied with the total number of lymphocytes in full blood. NK cells were hardly detectable until the start of G-CSF-treatment (arrow). Sampling month and year are indicated.

were tested in a chromium release assay, and no NK activity (assessed as specific lysis of K562) was demonstrated (Fig. 4). These studies were performed at different time points, but no NK cell cytotoxicity emerged after the slight increase in NK cell counts (data not shown). After incubation with IL-2, however, cytolytic activity increased dramatically against both targets tested, with specific lysis approaching 50% even at E/T = 5/1 (Fig. 4), indicating that LAK cell precursors were abundant in the patient's peripheral blood. Cytotoxicity was partially dependent on LFA-1, as demonstrated in the blocking experiments, but apparently independent of LFA-2 and VLA-4 (Fig. 3).

Spontaneous T cell cytotoxicity, tested in the redirected system, showed T cell cytolytic activity within the normal range, although in the lower end (data not shown).

## DISCUSSION

We report a patient with a primary immunodeficiency, where a lack of NK cells appears to be an integral part. In spite of his

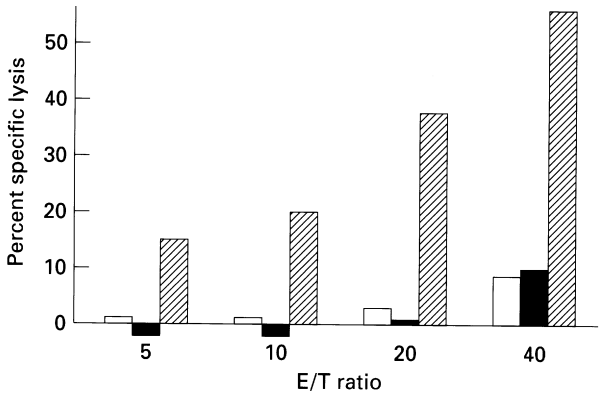


**Fig. 3.** The patient's mononuclear cells had no spontaneous cytolytic activity. No cytolytic activity was demonstrated in freshly isolated mononuclear cells from the patient, as determined in a 4-h chromium release assay: target cells were the Daudi (□) and the K562 (■) cell lines. Health blood donor control natural killer (NK) activity against K562 was determined in parallel (▨).

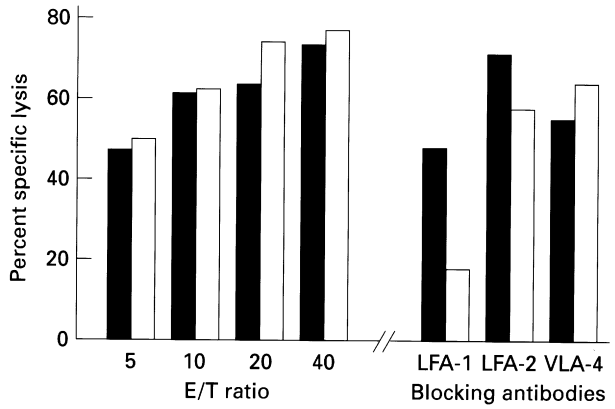


**Fig. 4.** The patient had lymphokine-activated killer (LAK) cell precursors, which were partially dependent on LFA-1. LAK cell activity was induced by culturing the mononuclear cells from the patient for 5 days in medium supplemented with 100 U/ml IL-2. A powerful cytolytic activity against both Daudi (□) and K562 (■) targets was demonstrated, even at low effector/target cell (E/T) ratios. The right side of the graph shows the effector cell dependency on various adhesion molecules. Effector cells were pre-incubated for 30 min with MoAbs against different adhesion molecules, before they were tested in the chromium release assay (E/T ratio = 20/1).

NK cell deficit, this young boy did well, with no serious infections after the start of immunoglobulin substitution, until he developed neutropenia. This would suggest that NK cells are of minor importance in the immune response to viral infections, at least in small children. However, the NK-cell deficient patient described by Biron *et al.* [11] had her first life-threatening infection much later in life, at the age of 13 years. There are also other differences between this patient and our patient. In both, NK cell function was hardly detectable in freshly isolated mononuclear cells, but only in our patient was full scale LAK activity demonstrated after 5 days culture with IL-2. This shows that LAK cell precursors are abundantly present in the peripheral blood of this patient, in contrast to the patient of Biron *et al.* [11]. The LAK phenomenon has generally been associated with either of two subsets, namely the NK-derived $CD3^-CD56^+$ or the $CD3^+CD56^+$ (double-positive) T cell subset [5,17]. Our patient clearly demonstrates LAK activity in a third subset, namely the $CD3^+CD56^-$ population. This potential may also in part explain the advantageous course of the disease in our patient.

Both NK cells and T cells can lyse their targets by releasing cytoplasmic, lytic granules [18,19]. T cells may alternatively utilize the fas-mediated pathway, where membrane protein (fas/fas-ligand) interactions convey signals leading to the induction of DNA fragmentation and apoptosis in the target cells [20,21]. Fas-ligand is only expressed in the membrane of activated T cells [21], and the LAK cell cytotoxicity observed in our patient can thus be accounted for by this alternative effector mechanism.

The number of well characterized immunodeficiency syndromes is rapidly increasing, and some of them include NK cell defects [10]. Isolated NK cell deficiencies are, however, extremely rare [3,10], and although not previously described, it seems reasonable to assume that it is an integral part of the hyper-IgM syndrome in our patient. This association is not generally present, as demonstrated by the normal NK cell number and function found in three other patients with hyper-IgM syndrome. This difference may be explained by the particular mutation in the CD40L gene found in our patient.

As mentioned, NK cells are probably developmentally related to T cells [7], but they also share a number of surface antigens and functional properties with myeloid cells [3]. The pathogenesis of hyper-IgM-associated neutropenia seen in approximately half of these patients is unclear [22]. The expression of CD40L on activated T cells is necessary for the terminal differentiation of B cells, but is also shown to have effects on monocytes and T cells, being implicated in growth, differentiation and cytokine production [23]. Thus, CD40L has pleiotropic biological effects and may very well be involved, directly or indirectly, in the generation of both NK cells and granulocytes.

The successful treatment of the hyper-IgM-associated neutropenia with G-CSF has recently been reported by another group [22]. The cytokine network is characterized by redundancy, and it is not unlikely that the lack of appropriate signals mediated through the cytokine-like CD40L molecule [24] can be bypassed by exogenous G-CSF. This cytokine exerts its effects by blocking apoptotic cell death, rather than stimulating proliferation [25], quite similar to how signals mediated by CD40L-expressing T cells rescue B cells from apoptosis in germinal centres [14].

We know little about how colony-stimulating factors act on NK cells and LAK cell precursors *in vivo*. However, while granulocytic-macrophage colony-stimulating factor (GM-CSF) has been reported to exhibit inhibitory effects on the generation of NK cells *in vitro*, no such effect was shown for G-CSF [26]. By contrast, a recent study of G-CSF-mobilized peripheral blood progenitor cells for allogeneic transplantation demonstrated a marked effect on NK cell progenitors, showing as much as 20 times more NK cells in the graft compared with untreated controls [27].

In conclusion, we have presented a patient with a hyper-IgM syndrome combined with NK cell deficiency. The latter has apparently only minor clinical implications, probably due to the demonstrated T cell rescue of NK cell function. Thus, this is still another example of the complex redundancy of the immune response in humans, represented by an immunodeficient patient.

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