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Decreased natural killer cell activity in late-onset hypogammaglobulinaemia

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SUMMARY

1. Natural killer cell activity and monocyte cytotoxicity was evaluated in three subgroups of patients with primary hypogammaglobulinaemia (ten patients with late-onset, eight with X-linked and five with early-onset disease) and in two patients with secondary late-onset hypogammaglobulinaemia against the K-562 erythro-leukaemia, the CaCo-2 colon carcinoma and the HGT-1 gastric carcinoma cell lines and compared with the results found in healthy control subjects.

2. The natural killer cell activity, both spontaneous and after stimulation with recombinant γ-interferon, was found to be decreased in patients with late-onset hypogammaglobulinaemia. The natural killer cell activity in this subgroup was found to be impaired in 60% of the patients \( (P<0.05) \). Within the other forms of primary hypogammaglobulinaemia a decreased natural killer cell activity was found to be less frequent (33%).

3. The lectin-mediated cytotoxicity by phytohaemagglutinin resulted in a similar maximal cytotoxicity in patients and control subjects.

4. The cytotoxicity of monocytes, spontaneous and after recombinant γ-interferon stimulation, was found to be normal in all patients with hypogammaglobulinaemia.

5. The impaired natural killer cell activity which was found in patients with late-onset hypogammaglobulinaemia may contribute to the increased susceptibility to infections and to the increased incidence of malignancies in this subgroup of patients with primary hypogammaglobulinaemia.

Key words: cytotoxicity, hypogammaglobulinaemia, γ-interferon, monocytes, natural killer cells.

Abbreviations: \( \text{r} \gamma \text{-IFN} \), recombinant \( \gamma \)-interferon; NK, natural killer; PHA, phytohaemagglutinin.

INTRODUCTION

Primary hypogammaglobulinaemia, especially in its late-onset form, is associated with impairments of not only humoral but also of cellular immune responses [1, 2]. Several studies indicated that these patients have an increased incidence of malignancies; in particular, patients with late-onset hypogammaglobulinaemia have an increased chance of developing non-Hodgkin lymphoma and a 50-fold increased chance of developing stomach cancer [3–5]. Natural killer (NK) cells are part of the natural immune system and play a role in the resistance against virus-infected cells and tumour cells [6]. Studies on NK cell activity in primary immunodeficiencies have been scarce and inconclusive, i.e. both normal and incidentally decreased NK cell activity have been found. However, a majority of the studies indicate that within the group of patients with common variable hypogammaglobulinaemia some patients show decreased NK cell activity, but in this regard no subpopulations of patients could be defined (Table 1 [7–13]).

In the present study we measured the NK cell activity and monocyte cytotoxicity in well-defined subgroups of patients with primary hypogammaglobulinaemia. Cytotoxicity, both spontaneous and after stimulation with recombinant \( \gamma \)-interferon and the lectin phytohaemagglutinin (PHA), was determined against lymphoid and gastrointestinal cell lines.

MATERIALS AND METHODS

Patients and control subjects

Twenty-three patients with primary hypogammaglobulinaemia were studied. Ten patients with late-onset hypogammaglobulinaemia (four females and six males,
mean age 38 years, range 17–68 years), eight patients with X-linked hypogammaglobulinemia (eight males, mean age 25 years, range 17–37 years), five patients with early-onset hypogammaglobulinemia (one female and four males, mean age 24 years, range 19–31 years) and two patients with secondary hypogammaglobulinemia due to a lymphoproliferative cancer (two males with multiple myeloma, mean age 67 years, range 57–78 years). All patients had reduced or absent serum γ-globulin and/or administration of antibiotics.

The criteria used for the diagnosis of primary hypogammaglobulinemia were for X-linked hypogammaglobulinemia the onset of clinical signs during the first 2 years of life, a family history compatible with an X-linked pattern of inheritance, and absence or profound decrease of B-lymphocytes in the peripheral blood or bone marrow. In patients in whom clinical symptoms began before the age of 2 years but no evidence for X-linked inheritance was found, the diagnosis of early-onset hypogammaglobulinemia was made. In these patients, B-lymphocytes were either absent, scarce or present in normal numbers. Patients were considered to have late-onset hypogammaglobulinemia if clinical manifestations began after the age of 10 years and B-lymphocytes, but not plasma cells, were found in the bone marrow [1,2].

The control subjects consisted of 25 healthy volunteers (nine females and 16 males, mean age 36 years, range 22–58 years), none of whom had signs of immunodeficiency or recurrent infection.

### Peripheral blood mononuclear cells

Mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation of heparinized peripheral blood. Part of the total mononuclear cell suspension was used in the cytotoxicity assay without further purification, while the other part was separated in lymphocytes and monocytes by elutriation centrifugation by a previously described technique [13]. The viability of these fractions, determined by Trypan Blue exclusion, was more than 98%. The fractions were kept in RPMI 1640 medium supplemented with 2 mM-glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml), gentamycin (50 µg/ml), amphotericin B (2.5 µg/ml) and 10% (v/v) fetal calf serum. The composition and purity of the cell fractions were evaluated by cell identification on cytospins with a May-Grünwald/Giemsa stain and non-specific esterase staining for monocytes in the cell suspensions. The purity of the lymphocyte fraction was usually above 98%, whereas for monocytes this was found to be greater than 80%. Monocyte purity was further increased to above 95% by 90 min adherence in 96-well culture plates and removal of the non-adhered cells by three washings with culture medium.

### Cytotoxicity assay

The cytotoxicity assay was performed as previously described [14]. Effector cells, i.e. total mononuclear cells, and separated lymphocytes and monocytes were cultured for 24 h at 37°C in a 5% CO2/95% air mixture in culture medium to reverse any possible adverse effects of the isolation process before functional assay. The target cells used were the erythroleukaemia cell line K-562, the human colon cancer cell line CaCo-2 and the human gastric carcinoma cell line HGT-1 [15]. The K-562 and HGT-1 cell lines were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, CaCo-2 with 20% (v/v) fetal calf serum and antibiotics, and were kept in growth phase by transferring 1:3 twice a week.

The target cells were harvested and labelled with Na251CrO4 (100 µCi/5 x 10⁶ cells; NEN Dupont, West Germany) for 1 h at 37°C. The effector to target ratio used for all populations was 50:1, for the monocytes the number of plating cells was adjusted to the 2.5 x 10⁶/ml non-specific esterase positive cells. Recombinant γ-interferon (γ-IFN; Boehringer, Ingelheim, West Germany) and PHA (Wellcome Research, Beckenham, Kent, U.K.) were dissolved in culture medium. The assay concentration for γ-IFN was 500 units/ml added at the start of the 24 h preincubation period. The concentration of PHA was 1 µg/ml. The assay was performed in triplicate with a final volume of 200 µl/well. After the target cells were added, the plates were centrifuged for 5 min at 500 g and incubated for 18 h at 37°C in a 5% CO2/95% air atmosphere. After 18 h incubation the supernatant was

### Table 1. NK cells in hypogammaglobulinemia

<table>
<thead>
<tr>
<th>Abbreviations: CVH, common variable hypogammaglobulinemia; IgA def, selective IgA deficiency; XLA, X-linked agammaglobulinemia; CVA, common variable agammaglobulinemia; CVI, common variable immunodeficiency; SCID, severe combined immunodeficiency; Ig, immunoglobulin.</th>
<th>Reference</th>
<th>Immunodeficiency (no. of patients)</th>
<th>NK cell observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Siriani et al. [7]</td>
<td>IgA def (10), CVH (5), XLA (3)</td>
<td>Normal activity</td>
<td></td>
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<tr>
<td>Koren et al. [8]</td>
<td>CVI (6), XLA (5)</td>
<td>Normal activity</td>
<td></td>
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<tr>
<td>Messina et al. [9]</td>
<td>CVI (31)</td>
<td>Normal to decreased activity; no subgroups</td>
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<tr>
<td>Gupta [10]</td>
<td>CVI (8), IgA def (4), XLA (1)</td>
<td>Decreased numbers (eight out of 13 patients)</td>
<td></td>
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<tr>
<td>Lipinski et al. [11]</td>
<td>CVI (5), XLA (2), IgA def (1)</td>
<td>Decreased activity (50% of patients)</td>
<td></td>
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<tr>
<td>Peter et al. [12]</td>
<td>SCID, low Ig levels (8)</td>
<td>Decreased activity (six out of eight patients)</td>
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<tr>
<td>Clerici et al. [13]</td>
<td>CVH (1)</td>
<td>Decreased activity</td>
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collected by a harvesting system (Supernatant Collection System; Skatron AS, Lier, Norway). The released label was counted in a γ-counter. The spontaneous release was determined by incubation of the target cells with culture medium, rγ-IFN or PHA and the maximal release by incubation of the targets with saponin. Cellular cytotoxicity was calculated by using the following formula:

\[
\text{Cytotoxicity (\%) = } \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100
\]

The cytotoxicity assay for all cell populations, i.e. total mononuclear cells and the enriched lymphocytes and monocytes, was performed uniformly as an 18 h assay. The specificity of the 18 h cytotoxicity assay for NK cells was determined by complement lysis of Leu 11 (CD16) monoclonal antibody coated mononuclear cells in four experiments. Inhibition of the 18 h cytotoxicity was above 85% both for K-562 and for CaCo-2.

**Statistical analysis**

All data are expressed as means ± sem. The statistical significances of the differences were evaluated with the non-parametric two-tailed Wilcoxon's rank-sum and the χ²-Fisher's exact tests.

**RESULTS**

The spontaneous NK cell activity showed a tendency to be decreased in the total group of patients with primary hypogammaglobulinaemia only against the K-562 targets \((P=0.056)\). When divided into subgroups, only patients with late-onset hypogammaglobulinaemia were found to have a significantly impaired NK cell activity \((P<0.05)\) against the K-562 targets; the NK cell activity against the CaCo-2 and HGT-1 was similarly impaired, although the differences failed to reach statistical significance (Table 2). When 50% cytotoxicity was used as the lower limit of normal, 60% of the patients with late-onset hypogammaglobulinaemia (six out of ten) were found to have low levels of NK cell activity \((P<0.05)\), whereas in X-linked (four out of eight; 50%), in early-onset (one out of five; 20%) and in secondary late-onset hypogammaglobulinaemia (none out of two; 0%) NK cell activity was not significantly different when compared with the control subjects (one out of 19; 5%) (Fig. 1). No differences were found in any of the clinical parameters (age, sex, duration of illness and treatments) between patients with decreased NK cell activity and those without.

Lectin-mediated cytotoxicity by PHA resulted in a significant increase in the cytotoxicity of the total mononuclear cells of all patients and control subjects (all groups \(P<0.05\)), irrespective of the underlying disease or the target cell line used, towards a maximal cytotoxicity level of 75–80%.

rγ-IFN also stimulated the NK cell activity substantially (all groups \(P<0.05\)). However, this stimulation did not overcome the deficient spontaneous cytotoxicity found in the patients with late-onset hypogammaglobulinaemia (Table 2).

Studies with the enriched lymphocytes from patients and control subjects, obtained by elutriation centrifugation, showed a similar pattern in the spontaneous and stimulated cytotoxicity revealing a lower NK cell response in patients with late-onset hypogammaglobulinaemia (data not shown).

Purified monocytes, both from patients and control subjects, hardly showed any spontaneous cytotoxic activity against each of the cell lines used. Stimulation

| Table 2. NK cell activity, spontaneous and after stimulation with rγ-IFN, of total mononuclear cells from patients with hypogammaglobulinaemia and control subjects |
|---------------------------------------------|-------------------|------------------|------------------|
| Results are means ± sem with \( n \) in parentheses. Statistical significance: \* \( P=0.056 \), \*\* \( P<0.05 \) compared with controls. |
|                           | Cytotoxicity (%)   |
|                            | K-562  | CaCo-2 | HGT-1  |
| **Spontaneous**            |        |        |        |
| Controls                   | 64.0 ± 3.2(19)   | 38.6 ± 4.5(23) | 53.2 ± 6.7(13) |
| Hypogammaglobulinaemia     |        |        |        |
| Primary (total)            | 54.1 ± 3.8*(23)  | 35.0 ± 3.8(19) | 42.9 ± 4.2(22) |
| Late-onset                 | 49.6 ± 6.1***(10)| 27.0 ± 5.1(7) | 36.8 ± 7.1(9)  |
| Early-onset                | 66.4 ± 7.7(5)    | 41.6 ± 9.6(5) | 53.2 ± 9.7(5)  |
| X-linked                   | 52.0 ± 5.5(8)    | 38.3 ± 5.7(7) | 43.4 ± 5.8(8)  |
| Secondary                  | 71.5 ± 6.5(2)    | 57.6 ± 15.9(2)| 61.7 ± 17.8(2) |
| **Stimulation with rγ-IFN**|        |        |        |
| Controls                   | 71.4 ± 2.4(19)   | 55.2 ± 4.7(20) | 64.6 ± 5.5(13) |
| Hypogammaglobulinaemia     |        |        |        |
| Primary (total)            | 61.0 ± 3.4***(22)| 47.3 ± 4.5(18) | 55.5 ± 4.5(21) |
| Late-onset                 | 57.4 ± 5.2***(9)| 36.6 ± 5.3***(6)| 49.1 ± 7.1(8) |
| Early-onset                | 69.7 ± 7.0(5)    | 52.4 ± 10.6(5) | 60.2 ± 10.5(5) |
| X-linked                   | 59.6 ± 6.0(8)    | 52.8 ± 7.2(7) | 58.9 ± 7.5(8)  |
| Secondary                  | 73.4 ± 3.6(2)    | 70.9 ± 12.3(2) | 73.3 ± 8.8(2)  |
with rγ-IFN increased the monocyte-mediated cytotoxicity significantly (all groups \( P < 0.05 \)) with no differences between patients, subgroups or patients and control subjects, nor between the three target cell lines used (Table 3).

**DISCUSSION**

In the present study we evaluated the NK cell activity in patients with primary hypogammaglobulinaemia. Patients with late-onset hypogammaglobulinaemia were found to have a significant decreased NK cell activity, both spontaneous and after stimulation with rγ-IFN, whereas such an impairment was less prominent in patients with other forms of primary hypogammaglobulinaemia and absent in secondary hypogammaglobulinaemia. Previous reports on NK cell activity in primary hypogammaglobulinaemia were not conclusive with regard to disease-related differences. Some studies describe a normal NK cell activity [7, 8], whereas others show decreased NK cell activity or NK cell numbers in hypogammaglobulinaemia patients without the identification of subgroups of patients [9-13]. In our study we also found a normal NK cell activity in the total group of patients with primary hypogammaglobulinaemia. Remarkably, however, both Gupta [10] and Lipinski et al. [11] found that in the subgroup of late-onset hypogammaglobulinaemia patients NK cell numbers or activity were low (respectively three out of eight, and two out of four patients). We found similar numbers of patients with decreased NK cell activity in the subgroup of patients with late-onset hypogammaglobulinaemia (six out of ten patients). In the patients with other forms of hypogammaglobulinaemia, a decrease in NK cell activity was found less frequently in our study, which corresponds with previous reports [7, 8]. However, the number of patients studied were small which limited statistical analysis of the frequency of this phenomenon in these subgroups.

The decrease in NK cell activity of late-onset hypogammaglobulinaemia patients in our study was not target specific, since it was noticed against the standard NK cell line K-562 but also against the colorectal cancer CaCo-2 and the gastric cancer HGT-1 cell lines. This is especially interesting since patients with late-onset hypogammaglobulinaemia have an increased incidence of non-Hodgkin lymphoma and gastric cancer [3, 4]. Thus, the decreased NK cell activity may identify late-onset hypogammaglobulinaemia as a pre-malignant condition. Interestingly, a completely different study by our group on the gastrin response to bombesin stimulation in primary hypogammaglobulinaemia, also identified the late-onset hypogammaglobulinaemia patients as a high cancer risk subgroup [16]. Prospective and follow-up studies need to be performed to determine whether the susceptibility for malignancy is especially increased in the patients with reduced NK cell activity and reduced plasma gastrin response to bombesin stimulation.

We found a normal response of the NK cells to rγ-IFN which did not overcome the decreased spontaneous NK cell activity in the patients with late-onset hypogammaglobulinaemia. These findings correspond to previous reports in which both the production of and the response to interferon in hypogammaglobulinaemia patients was found to be normal [9, 11].

Hypogammaglobulinaemia patients showed a normal lectin-mediated cytotoxicity with no difference between the subgroups. There is only one other study, by Lubens et al. [17], which showed that two patients with hypogammaglobulinaemia also had a normal PHA-induced cytotoxic response, therefore indicating that the synthesis of interleukin-2 and its receptor, which is mediated by PHA, is fully intact in patients with primary hypogammaglobulinaemia.

Monocytes, known to have cytotoxic capabilities [18], showed a normal spontaneous and rγ-IFN-induced cytotoxicity in patients with primary hypogammaglobulinaemia.
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which indicates that they do not contribute to the deficient cytotoxic response of patients with late-onset hypogammaglobulinemia.

In conclusion, a majority of patients with late-onset hypogammaglobulinemia have an impaired NK cell activity against several tumour cell lines. Stimulation with γ-IFN and PHA results in a normal response in these patients. Other forms of primary hypogammaglobulinemia have a lower frequency of the impairment of NK cell activity. The relevance of these findings with regard to the high incidence of gastric cancer in late-onset hypogammaglobulinemia has yet to be established.

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