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Immunoglobulin D enhances the release of tumour necrosis factor-α, and interleukin-1β as well as interleukin-1 receptor antagonist from human mononuclear cells

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SUMMARY

Immunoglobulin D (IgD) is normally present in only low concentrations in serum. In the hyper-IgD and periodic fever syndrome (HIDS), however, serum levels exceed 140 mg/l. This syndrome is further characterized by recurrent inflammatory febrile attacks together with an acute phase response and appearance of cytokines in the circulation. The role of IgD in the pathogenesis of HIDS and its relation to the increased cytokine concentrations is unclear. Therefore, we tested whether IgD, IgG and α 1-acid glycoprotein (AGP) isolated from human serum influence the synthesis of interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), and IL-1ra, as measured by specific radioimmunoassays, in human peripheral blood mononuclear cells (PBMC). Incubation of PBMC with IgD and AGP for 24 hr led to increased release of IL-1β, TNF-α, and IL-1ra. The magnitude of stimulation of IgD exceeded that of AGP; the effect by IgD was dose-dependent and showed a 30-fold (TNF-α) to almost 150-fold (IL-1β) increase at the highest concentration (50 mg/l), while AGP (750 μg/ml) only increased the cytokine secretion fourfold (TNF-α) to almost 30-fold (IL-1β). The effect of IgD on IL-1ra was less dramatic but a fivefold increase was observed at 50 mg/l compared with a 2.5-fold increase with AGP. IgD potentiated the effect of lipopolysaccharide (LPS) on secretion of both IL-1β and TNF-α, although the effect was most apparent for TNF-α. Apart from inducing IL-1ra synthesis, IgG did not influence cytokine release in human PBMC. These data indicate that IgD is a potent inducer of TNF-α, IL-1β and IL-1ra and thus may contribute to the pathogenesis of HIDS.

INTRODUCTION

Immunoglobulin D (IgD) was first discovered by Rowe & Fahey in 1965 and it is the major antigen receptor isotype coexpressed with IgM on the surface of human peripheral B cells. Its biological function has not been fully elucidated, but IgD may be involved in the early antibody response by augmenting antibody secretion. In serum, IgD is present only in minor quantities and it constitutes only some 0.2% of all immunoglobulins. In normal individuals concentrations vary from undetectable to 140 mg/l. Elevated serum IgD may be found in several clinical conditions such as IgD myeloma, Hodgkin’s disease, sarcoidosis and tuberculosis, aspergillosis, ataxia-telangiectasia, acquired immune deficiency syndrome and the hyperimmunoglobulinemia D and periodic fever (HIDS) syndrome. The latter clinical entity is characterized by recurrent febrile attacks with abdominal complaints, arthralgias/arthritis, skin lesions and an elevated serum IgD concentration beyond 140 mg/l. The serum concentration of IgD remains elevated throughout life and does not change with disease activity. The attacks are accompanied by an acute phase response with high circulating concentrations of α 1-acid glycoprotein (AGP), C-reactive protein (CRP), soluble type II phospholipase A₂ and serum amyloid A. Inflammatory cytokines such as interleukin-1β (IL-1β), IL-6 and tumour necrosis factor-α (TNF-α) are thought to play a central role in multiple effector functions and cellular interactions in inflammation and immune response. Uncontrolled secretion leading to high levels of inflammatory cytokines may lead to an intense inflammatory response as noted in HIDS. Indeed, elevated circulating concentrations of the inflammatory mediators TNF-α, IL-1 receptor antagonist (IL-1ra), IL-6, interferon-γ, and soluble receptors for TNF-α (sTNF-R) have been detected during the febrile episodes.

Upon stimulation with bacterial lipopolysaccharide (LPS, endotoxin), monocytes/macrophages are able to produce a wide array of cytokines such as IL-1β, IL-6 and TNF-α. During attacks of HIDS, incubation of whole blood with LPS for 24 hr, resulted in an augmented ex vivo secretion of TNF-α,
IL-1β and IL-1α in the supernatants compared with convalescence.\textsuperscript{6} Taken together, these data suggest that inflammatory cytokines such as IL-1β, IL-6 and TNF-α could play a pathogenic role in the febrile attacks of the hyper-IgD syndrome. Furthermore, it can be speculated that soluble compounds present during an attack are responsible for this up-regulated cytokine secretion. For example, the acute phase protein CRP is able to potentiate the secretion of IL-1β, IL-1α and TNF-α by isolated human monocytes.\textsuperscript{11} On the other hand, the effect of IgD on cytokine secretion is not known. These observations prompted us to investigate the capacity of serum IgD, IgG and AGP to modulate the release of cytokines in human mononuclear cells.

**MATERIALS AND METHODS**

**Sera and plasma**

Serum for IgD isolation was obtained from a patient with the hyper-IgD syndrome (patient represents no. 1 in a recent review).\textsuperscript{5} During the 13-year follow-up (1981–94) she had IgD concentrations varying from 2269 mg/l to 7484 mg/l. Plasma was stored at −20°C after the addition of E-aminocaproic acid (final concentration 0.5%) to prevent ‘spontaneous’ degradation of IgD.\textsuperscript{12} Sampling of experimental plasma was performed during remission, defined as the absence of symptoms for at least 2 weeks. The patient did not use any medication at the time of the study.

**Enzyme-linked immunosorbent assay for IgD**

Determination of IgD was performed using a specific enzyme-linked immunosorbent assay (ELISA). Microtitre plates (Nunc, Roskilde, Denmark) were directly coated for the titration of human IgD with 1:5000 dilution of rabbit anti-human IgD in 200 μl bicarbonate buffer, pH 9.5 (Dako, Copenhagen, Denmark). As there was no non-specific binding detectable in our assay, we did not block the plates with albumin. After overnight incubation at 4°C, plates were washed with phosphate-buffered saline (PBS) containing 0.01% Tween 20, and samples were added to the wells. Plates were incubated again overnight at 4°C. A standard human serum (Behring, Marburg, Germany), calibrated against the British Research Standard No 67/37, was used as a reference.\textsuperscript{13} The plates were washed and 100 μl mouse monoclonal anti-human IgD, diluted 1:200 in PBS, was added and incubated for 90 min at 37°C. After a washing step, 100 μl horseradish peroxidase rabbit anti-mouse anti-IgD (diluted 1:4000 in PBS) (Dako, Copenhagen, Denmark) was added and incubated at 37°C for 90 min.

After a washing step, IgD was measured by incubation with 100 μl ortho-phenylenediamine (Sigma, St Louis, MO). The reaction was stopped by 4 μl H₂SO₄ and absorbance was read at 492 nm using a Titertek Multiskan ELISA reader (Eflab, Oy, Helsinki, Finland). The lower limit of detection was 1.4 mg/l.

**Preparation and purification of IgD**

IgD was prepared from hyper-IgD sera by chromatography on diethylaminoethyl (DEAE)–cellulose and CM-C50 columns followed by gel filtration on Sephacryl S-300. All steps were performed at 4°C. Serum was precipitated with polyethylene-glycol (PEG) 6000 (final concentration 7%). The precipitate was then dissolved in 0-01 M trishydroxymethylaminomethane (Tris), 2 mM ethylenediamine-tetraacetic acid (EDTA), 1 mM phenylmethysulphonyl fluoride (PMSF), and finally 0-5 U/ml aprotinin, pH 7.8 to prevent proteolysis. The solution was applied to a column of DEAE–cellulose (1.5 cm × 60 cm) equilibrated in the same buffer. The column was eluted with a linear gradient (0.01 – 0.05 M) of the Tris buffer. IgD-containing fractions were pooled, dialysed against 0.01 M sodium acetate, 2 mM EDTA, 1 M PMSF and 0.5 U/ml aprotinin, pH 6.0 and applied to a CM-C50 column (2.5 cm × 15 cm) equilibrated in the same buffer. The IgD-positive fractions were again pooled and finally applied to a Sephacryl S-300 column (2.5 cm × 90 cm) equilibrated with a buffer containing veronal buffered NaCl, 2 mM EDTA, 0.15 M NaCl, 1 M PMSF and aprotinin, pH 7.4. The purity was checked by sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis. The final IgD product contained 150 mg/l IgD as measured by the described ELISA and no detectable IgG, IgA or IgM as detected by immunoturbidimetric assay (Cobas Fara, Roche Diagnostics, Basle, Switzerland). The IgD preparation was dissolved in PBS and stored at −70°C until use.

The purified IgD preparation was tested for endotoxin using the limulus amoebocyte lysate assay (Sigma) and was shown to contain less than 6 pg/ml endotoxin.

**SDS-PAGE and detection of proteins on membranes**

The purity was checked by SDS–polyacrylamide gel electrophoresis (PAGE) following the method of Laemmli.\textsuperscript{14} Briefly, the IgD product was subjected to SDS–PAGE and proteins were stained with silver nitrate using a PhastSystem development unit (Pharmacia, Uppsala, Sweden). The silver staining is able to detect 0.3 ng/μl protein.\textsuperscript{15} Proteins were blotted on nitrocellulose and the blots were incubated with a polyclonal anti-IgD antibody (DAKO A093, Dako, Copenhagen, Denmark). After a washing step a specific secondary biotinylated antibody was added. Bands were visualized with a streptavidin complex and photographed.

**Preparation of IgG**

Purified human serum IgG preparation (Globuman Berna) was kindly provided by Primmned BV, the Netherlands. The preparation contained more than 95% IgG and no IgA and IgM as examined by an automatic immunoturbidimetric assay (Cobas Fara, Roche Diagnostics, Basle, Switzerland) and no IgD as assessed with ELISA. The IgG preparation was dissolved in PBS and stored at −70°C until use.

**Preparation and purification of AGP**

AGP obtained from Cohn fraction V of pooled human serum was a gift of Drs E. Havenaar and W. van Dijk, Free University of Amsterdam, the Netherlands. Purification was performed following the method of Hao and Wickerhauser using two purification steps with absorption on DEAE–Sephadex and chromatography on a CM cellulose column.\textsuperscript{16} The purity was checked by SDS–PAGE and by crossed immunoelectrophoresis using rabbit anti-human serum and rabbit anti-human AGP antisera (Dako, Copenhagen, Denmark). The final preparation was dissolved in PBS and stored at −70°C until use.

**Isolation of peripheral blood mononuclear cells (PBMC)**

Peripheral blood from healthy, medication-free donors (25–45
years of age) was drawn into sterile 10-ml tubes containing (0.2 mg EDTA. PBMC were isolated by buoyant density gradient centrifugation on Percoll.\textsuperscript{17} The cells from the interphase were aspirated and washed twice in sterile saline. After the last washing, the cells were resuspended at a concentration of $5 \times 10^6$/ml in RPMI-1640 medium (Dutch -2
determined.

After incubation for 24 hr, the culture plates were centrifuged (5%) was added after filtration (complete medium). PBMC

were aspirated and washed twice in sterile saline. After incubation for 24 hr, the culture plates were centrifuged at 4000 # for 10 min to remove cellular material. Then, the cell supernatants were aspirated and cytokine content was

Isolation of monocvtes by adherence
PBMC were suspended in complete medium at a concentration of $5 \times 10^6$/ml and allowed to adhere on 96-well plastic tissue culture plates (Greiner GMBH, Frickenhausen, Germany) and incubated at 37° in a humidified atmosphere containing 5% CO$_2$ for 24 hr. PBMC cultures set up in the presence of complete medium served as controls. After incubation for 24 hr, the culture plates were centrifuged at 4000 g for 10 min to remove cellular material. Then, the cell supernatants were aspirated and cytokine content was determined.

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Source of antiserum
Polyclonal antibodies for IL-1β were kindly provided by Selavo (Siena, Italy). Antibodies for TNF-α were a gift of Dr C. A. Dinarello (New England Medical Center, Boston, MA) and were obtained by immunizing New Zealand White rabbits with recombinant human TNF.\textsuperscript{19} A polyclonal anti-IL-1ra antiserum was raised in New Zealand White rabbits immunized with recombinant IL-1ra kindly provided by Synergen (Boulder, CO).\textsuperscript{20}

Radioimmunoassay for cytokines
IL-1β, TNF-α and IL-1ra in plasma were measured in duplicate by non-equilibrium radioimmunoassay (RIA) and 100 μl of rabbit polyclonal antibodies raised against recombinant cytokines as described elsewhere [final dilution IL-1β (1:210 000), TNF-α (1:150 000) and IL-1ra (1:60 000) which gives a binding with the tracer of approximately 25%]. The antibodies were dissolved in RIA buffer containing 13 mM Na$_2$EDTA, 0.02% sodium azide, 0.25% bovine serum albumin (Behring, Marburg, Germany), 0.1% Triton-X-100, 3 mM Na$_2$HPO$_4$, and 250 000 kallikrein-inactivating units apoprotinin (Bayer, Leverkusen, Germany) pH 7.4 were added to sample and standard.\textsuperscript{21} All reagents were prepared with this buffer. For circulating cytokine measurement 100 μl and for measurement of ex vivo cytokine production 25 μl (TNF-α) and 10 μl (IL-1β and IL-1ra) of sample and standard were added. The mixture was incubated for 1 day at room temperature. Subsequently, tracer (approximately 7000 d.p.m./100 μl) was added, and incubation was continued for another 2 days. To separate bound and free tracer from the free fraction, 100 μl of a separation agent (10% (vol/vol) sheep anti-rabbit immunoglobulin G and 0.01% [wt/vol] rabbit immunoglobulin G (Sigma)] was added to each tube and incubated for 30 min at room temperature. The antibody complex was completely precipitated by the addition of 1 ml 7.5% PEG-6000 (Merck, Darmstadt, Germany). For IL-1β, TNF-α and IL-1ra the range of the standard curve was 20 pg/ml to 3000 pg/ml. The sensitivity of the assay with 100 μl sample was 60 pg/ml (IL-1ra), 40 pg/ml (IL-1β) and 20 pg/ml (TNF-α). There was no cross-reactivity between IL-1ra and IL-1β (<0.001%), TNF-α and IL-1β (<0.01%) and IL-1ra and TNF-α (<0.01%). To minimize analytical errors, all samples were analysed in the same run, in duplicate.

Statistical analysis
All results are shown as the mean ± standard error of the mean (SEM). Cytokine concentrations were compared with the non-parametric Friedman two-way analysis of variance (ANOVA) and the unpaired non-parametric Mann–Whitney U test, where appropriate. A P value of <0.05 was considered to be the lowest level of significance.

RESULTS

SDS-PAGE analysis of IgD
IgD product was resolved by SDS-PAGE analysis on a 10% polyacrylamide gel to observe its integrity and purity. Silver nitrate staining showed a band of 185 000 MW indicating that the purified protein had not undergone aggregation or breakdown during isolation. Immunoblotting with polyclonal anti-IgD antibodies indicated that the isolated protein indeed consisted of IgD (Fig. 1).

Induction of IL-1β, TNF-α and IL-1ra synthesis in human PBMC and adherent monocytes by IgD and IgG
The induction of IL-1β, TNF-α and IL-1ra by IgD after a 24-hr incubation period is shown in Fig. 2. IgD was able to stimulate PBMC to a significant increase of the secretion of IL-1β, TNF-α (P < 0.0001), and IL-1ra (P < 0.005). Compared to results from control medium, a concentration as low as 0.05 mg/l IgD induced more IL-1β (1.01 ± 0.86 ng/ml versus 0.16 ± 0.03 ng/ml), IL-1ra (10.3 ± 1.91 ng/ml versus 2.4 ± 0.09 ng/ml), and TNF-α (1.09 ± 0.33 ng/ml versus 0.25 ± 0.08 ng/ml). Induction of IL-1β and TNF-α by IgD was dose-depended and continued to increase with the maximum concentration used (50 ng/l), while the stimulation of IL-1ra release did not increase appreciably with concentrations above 0.05 mg/l. The amount of IL-1β produced by PBMC at a concentration of 50 mg/l was nearly 150-fold the amount in control samples, while TNF-α secretion increased almost 30-fold. IgD at a concentration of 50 mg/l induced maximal IL-1ra secretion (13.1 ± 1.94 ng/ml). Incubation of IgD with
LPS (100 ng/ml) enhanced IL-1β and TNF-α release by PBMC ($P < 0.05$). Addition of IgD to LPS did not influence IL-1ra secretion from PBMC.

In order to delineate which cells were most responsive to IgD stimulation, monocytes were separated by adherence and cytokine secretion after 24 hr IgD stimulation was estimated. As shown in Table 1 monocytes secreted more IL-1β and TNF-α compared with non-adherent cells. This emphasizes the critical role of monocytes with regard to cytokine release in response to IgD stimulation in our system with a mixed cell population.

The time-course of IL-1β, TNF-α and IL-1ra production from PBMC in response to IgD (5 mg/l) was studied next and compared with that of LPS-stimulated PBMC (Fig. 3). Measurable amounts of IL-1β (0.28 ± 0.09 ng/ml), TNF-α (0.82 ± 0.04 ng/ml) and IL-1ra (0.76 ± 0.1 ng/ml) were detected after 2-hr incubation with IgD. After 2-hr and 4-hr incubation PBMC released more IL-1β but not TNF-α or IL-1ra with IgD than with LPS. Peak concentrations of TNF-α (4.38 ± 1.3 ng/ml) were already attained after 6-hr incubation with IgD and a non-significant decrease was observed after prolonged incubation (at 24 hr, 2.73 ± 0.48 ng/ml). After 12-hr incubation, both IL-1β (12.4 ± 1.56 ng/ml) and IL-1ra (7.8 ± 2.1 ng/ml) reached maximal concentrations with cytokine release from PBMC slightly diminishing after prolonged incubation.

To investigate the possibility that the effects observed with IgD were caused by endotoxin contamination, PBMC were incubated with polymyxin B (25 μg/ml). As shown in Table 2, polymyxin B did not influence the effect of IgD but gave a reduction of the LPS-induced IL-1β and TNF-α release. The incubation with polymyxin B did not influence the IL-1ra release by IgD or LPS. As an additional control for endotoxin contamination, the IgD preparation was heated in a polypropylene tube incubated in a water bath at 100° for 1hr. Exposure of PBMC to the IgD preparation resulted in

Table 1. Comparison of effect of a 24-hr incubation of IgD on TNF-α and IL-1β release (ng/ml) from PBMC, adherent monocytes and non-adherent cells

<table>
<thead>
<tr>
<th>IgD (mg/l)</th>
<th>0</th>
<th>0.05</th>
<th>0.5</th>
<th>5</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TNF-α release</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBMC</td>
<td>0.08</td>
<td>0.2</td>
<td>7.4</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Adherent monocytes</td>
<td>0.13</td>
<td>0.05</td>
<td>4.1</td>
<td>6.95</td>
<td>10</td>
</tr>
<tr>
<td>Non-adherent cells</td>
<td>0.08</td>
<td>0.125</td>
<td>2.25</td>
<td>3.95</td>
<td>1.7</td>
</tr>
<tr>
<td><strong>IL-1β release</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBMC</td>
<td>0.085</td>
<td>0.5</td>
<td>3.3</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Adherent monocytes</td>
<td>0.075</td>
<td>0.4</td>
<td>2.05</td>
<td>16.5</td>
<td>25.5</td>
</tr>
<tr>
<td>Non-adherent cells</td>
<td>0.065</td>
<td>0.4</td>
<td>0.65</td>
<td>2.95</td>
<td>4</td>
</tr>
</tbody>
</table>
Enhancement of cytokine secretion by IgD

Figure 3. Effect of time of addition of human IgD or LPS on IL-1β (a), TNF-α (b) and IL-1ra (c). PBMC were treated with human IgD (open blocks) (5 mg/l) or LPS (cross-hatched blocks) (100 ng/ml) at 2, 4, 6, 8, 12 or 24 hr cultures were harvested and cytokines were measured in the supernatants. Data are depicted as the mean ± standard error of five different donors; P < 0.005 when comparing PBMC treated with IgD compared with untreated PBMC.

substantial reduction of IL-1β (360 pg/ml) and TNF-α (100 pg/ml) release, to values comparable to those from cultures incubated without stimuli. This excludes the possibility that the stimulation by the IgD preparation was caused by endotoxin, which is relatively heat stable.

As shown in Fig. 4, human IgG was a potent stimulator for IL-1ra production (P < 0.005). As little as 0.05 mg/l IgG was able to release appreciable IL-1ra concentrations compared with baseline (8.1 ± 1.3 ng/ml versus 2.4 ± 0.17 ng/ml). The maximal effect was observed at concentrations of 0.5 mg/l, but the effect was smaller compared with IgD. In contrast, the presence of up to 50 mg/l IgG had little effect on IL-1β and TNF-α release in the same cultures. Also, IgG did not influence the LPS-induced cytokine production by PBMC.

Effect of complement-containing serum

Because C5a induces TNF-α and IL-1β messenger RNA from PBMC in vitro,22 we investigated the contribution of complement to the IgD-induced cytokine release. As the complete medium used in all previous experiments contained serum deprived of complement by heat inactivation (30 min at 57°C),

Table 2. PBMC were incubated for 24 hr. Data represent mean ± SEM from four experiments. Polymyxin B was used at a concentration of 25 μg/ml.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Inhibitor</th>
<th>IL-1β (ng/ml)</th>
<th>TNF-α (ng/ml)</th>
<th>IL-1ra (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgD (5 mg/l)</td>
<td></td>
<td>26 ± 3.1</td>
<td>4.7 ± 0.3</td>
<td>9.5 ± 1.7</td>
</tr>
<tr>
<td>IgD (5 mg/l)</td>
<td>Polymyxin B</td>
<td>21.5 ± 4.5</td>
<td>5.8 ± 1</td>
<td>9.8 ± 2.7</td>
</tr>
<tr>
<td>LPS (100 ng/ml)</td>
<td></td>
<td>31 ± 13</td>
<td>6 ± 1.6</td>
<td>8.35 ± 1.6</td>
</tr>
<tr>
<td>LPS (100 ng/ml)</td>
<td>Polymyxin B</td>
<td>11.4 ± 6.11</td>
<td>1.7 ± 0.57*</td>
<td>8.32 ± 2.1</td>
</tr>
</tbody>
</table>

*P < 0.05 from LPS alone.

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This study demonstrates for the first time that human serum enhanced in the presence of AGP, whereas no such effect was observed with non-treated complement-containing serum added to complete medium. This did not change the IgD-induced IL-1β, TNF-α and IL-1ra release from PBMC (data not shown.)

**Induction of IL-1β, TNF-α and IL-1ra synthesis in human PBMC by AGP**

AGP was also tested for its potential to elicit IL-1β, TNF-α and IL-1ra release from PBMC (Fig. 5). PBMC from five different donors incubated with AGP produced about two- to fourfold more cytokines compared with unstimulated controls (P < 0.05). The maximal effect of AGP on production of the various cytokines was observed with 750 µg/ml and higher concentrations did not yield different results. PBMC were also incubated with 100 µg/ml LPS and increasing concentrations of AGP. The LPS-stimulated synthesis of IL-1ra was significantly enhanced in the presence of AGP, whereas no such effect was detectable for TNF-α and IL-1β (P < 0.05).

**DISCUSSION**

This study demonstrates for the first time that human serum IgD enhances cytokine secretion from PBMC in vitro. The effect was dose-dependent and showed an almost 150-fold (IL-1β) and 30-fold (TNF-α) increase at the highest concentration of IgD used (50 µg/l). The effect on IL-1ra was less dramatic but a fivefold increase was observed at 50 µg/l. Moreover, IgD enhanced the effect of LPS on production of both IL-1β and TNF-α, although the effect was most apparent for TNF-α. These findings suggest a role of IgD in the pathogenesis of HIDS. IgD, being present in increased concentrations in these patients, could participate in the activation of the cytokine network during febrile attacks in these patients by the virtue of its cytokine-inducing capacity.

The cytokine-releasing effect of IgD is impressive and similar to that of LPS, which is regarded as one of the most potent biological stimuli for cytokine release from PBMC. It is unlikely that the effect of IgD is mediated via complement activation, as addition of complement-containing serum did not influence the cytokine release. Preliminary results also indicate that IgD is able to induce IL-6, IL-10, soluble TNF receptor p75, and leukaemia inhibitory factor release from PBMC (J. P. H. Drenth, unpublished observations). It is unlikely that the observed effects of IgD are a result of LPS contamination. First, we detected an endotoxin concentration of less than 6 pg/ml in our IgD stock solution (150 µg/l). In our experiments, IgD in a concentration of 0.05 µg/ml already induced cytokine secretion, and because this represents a dilution of 1:3000 of the stock solution this sample cannot contain over 0.002 pg/ml LPS. It has been shown that the LPS concentration must exceed 10 pg/ml in order to observe a significant induction of cytokine secretion from human PBMC. In addition, incubation with polymyxin B did not affect its cytokine-eliciting effects in PBMC.

Lastly, exposure of the IgD preparation for 1 hr to a temperature of 100°C resulted in complete disappearance of the cytokine-eliciting effect. Endotoxin degrades only after 4 hr exposure to 180°C.

Human IgD is a protein of MW 185 000 with a heavy chain consisting of four domains, an Fc region that is highly resistant to proteolysis, and a more labile Fab. It has been postulated that it may serve as a signalling mechanism to the T-cell population and, experimentally, it augments the antibody response to a variety of antigens. Peripheral blood CD4+ and CD8+ lymphocytes from healthy donors express receptors for IgD and are denoted as Tδ cells. In contrast to other immunoglobulin receptors, such as those for IgG, IgA, IgE and IgM, the IgD receptor is not specific only for the Fc region of IgD. There is strong evidence that murine IgD receptors are lectins, which bind not only to the Fcδ receptors but also to N-linked IgD-associated, carbohydrates. Exposure to oligomeric IgD and IL-2, IL-4 and IFN-γ results in up-regulation of these receptors. IgD may thus play a role in the regulation of immune responses via its recognition of these IgD-receptor-positive cells. Most probably, effective binding of IgD to these cells is needed to induce the cytokine-producing machinery and to explain the impressive and rapid cytokine release. Our data indicate that monocytes are the main producers of cytokines in the presence of IgD. Co-operation of lymphocytes and monocytes does not seem to play a role. It would be interesting to know whether IgD affects the transcription of mRNA for the various cytokines or that post-transcriptional mechanisms play a role. Further studies are underway to elucidate this issue. From our studies we cannot conclude...
whether the cytokine-inducing effect is specific for IgD isolated from hyper-IgD patients, or that it also applies to IgD isolated from other sources such as patients with IgD myeloma. The structure of IgD from both diseases however differs, and more than 90% of the IgD myelomas consist of lambda light chains, while the kappa/lambda light chain ratio is elevated in the hyper-IgD syndrome.\textsuperscript{29,30} Whether this structural difference results in a distinct cytokine-inducing effect will be addressed in a future study.

The cytokine-releasing effect of another immunoglobulin, IgG, has been investigated to some extent. Our finding of increased IL-1ra production with virtually absent production of pro-inflammatory cytokines in PBMC cultures incubated with IgG is in accordance with other studies.\textsuperscript{31} Culture of monocytes on adherent IgG specifically increases IL-1ra production but cells fail to release IL-1β.\textsuperscript{32} In our study, incubation with IgG failed to elicit either IL-1β or TNF-α secretion but enhanced IL-1ra release.

During the febrile attacks of the HIDS, a variety of acute phase proteins (serum amyloid A, and CRP) are produced and can be found in appreciable concentrations in the circulation. Moreover, during, but also between attacks, there are high circulating concentrations of AGP and the fucosylation of this protein dramatically changes during the febrile episodes.\textsuperscript{7} Incubation of AGP, in our study, resulted in a dose-dependent increase of release of IL-1β, TNF-α and also IL-1ra; the effect was most evident at a concentration of 750 μg/ml. Human AGP significantly enhanced the LPS-induced secretion of IL-1β but did not affect TNF-α and IL-1ra release. AGP has been shown to induce the synthesis of IL-1β and IL-1ra in human PBMC.\textsuperscript{33} In other studies AGP acted synergistically with low concentrations with LPS in the induction of IL-1β, TNF-α, IL-6 and IL-1ra.\textsuperscript{33,34} However, compared with IgD, the cytokine-enhancing effect of AGP is only moderate. During the acute phase response AGP undergoes extensive post-translational modification, with the acquisition of sialyl-Lewis-X containing glycans and shift of glycosylation with increase of biantennary glycans.\textsuperscript{7} We used AGP obtained from the Cohn fraction V of pooled human serum. It is possible that the induction of these cytokines depends on the extent of glycosylation of AGP, but this requires further investigation. Another acute phase protein, CRP, seems to be a more potent producer of IL-1β, IL-6, TNF-α and IL-1ra and also potentiates the LPS-induced secretion of these inflammatory mediators.\textsuperscript{11,33} All in all, these findings imply that the acute-phase proteins may serve an immunomodulatory role in attacks of the hyper-IgD syndrome by augmenting the cytokine release from PBMC.

From our results it is apparent that IgD stimulates human PBMC to secrete proinflammatory cytokines such as IL-1β but also of an inhibitor like IL-1ra. The amounts of IL-1β by far exceed those of IL-1ra. Excess concentrations of IL-1ra (100- to 1000-fold excess) must be present in order to yield a significant inhibition of IL-1-induced responses and thus the amount may be inadequate in the acute inflammation.

Although IgD is a potent stimulator of cytokines, its precise role in the pathogenesis of the syndrome remains to be clarified further. For example, a clear correlation between the serum concentration of IgD and the frequency or severity of the attacks is absent.\textsuperscript{5} However, it is possible that attacks in the hyper-IgD syndrome are precipitated by the inactivation of an as yet unidentified inhibitory compound that allows IgD to elicit an exaggerated cytokine response. In this respect IgD may function in a dual role, amplifying the inflammatory response by release of proinflammatory cytokines but also serving a down-regulating role by eliciting the production of IL-1ra. In this way IgD can act as a factor in maintaining the inflammatory response during the febrile attacks of the hyper-IgD syndrome.

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