In vitro effects of HA-1A (Centoxin) on cytokine production in whole blood from intensive care unit patients

S. M. Yentis, N. Soni and P. G. Riches

Summary

The cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-α (TNFα) have been implicated in the pathophysiology of sepsis and the systemic inflammatory response syndrome (SIRS). The anti-endotoxin antibody, HA-1A (Centoxin), introduced as a treatment for sepsis, was withdrawn because of possible toxicity in some patients. There has been little investigation of the effects of HA-1A on cytokine production. Sixty-one whole blood samples from 15 intensive care unit (ICU) patients with SIRS were incubated for 24 h with HA-1A and concentrations of cytokines determined. Concentrations of IL-6 exceeded those in samples incubated without HA-1A by more than 25% in five patients, of whom four died. One death occurred among 10 patients for whom IL-6 concentrations did not increase (P = 0.03). Incubation with HA-1A did not increase concentrations of IL-1β or TNFα. HA-1A did not affect cytokine production in whole blood from healthy subjects. HA-1A may induce IL-6 production in whole blood from some ICU patients and this response is associated with increased mortality. Immune therapies for treatment of sepsis and SIRS require careful evaluation of their ability to affect cytokine production, before they are introduced for general use. (Br. J. Anaesth. 1994; 73: 805–811)

Key words

Intensive care, Intensive care, infection. Polypeptides, cytokines.

Severe sepsis is a major cause of death in the intensive care unit (ICU) [1–3]. Recent attention has focused on the cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-α (TNFα) as major mediators of the systemic inflammatory response syndrome (SIRS) that occurs in sepsis [3–6]. Although gram-negative bacterial endotoxin is a potent initiator of cytokine production experimentally, its role in clinical sepsis is less clear; in particular, the clinical significance of circulating endotoxin is controversial [7]. However, encouraging initial experiments in animal models have led to the development of anti-endotoxin antibodies, one of which, HA-1A (Centoxin, Centocor), was released in Europe in 1991 [1, 8]. The clinical study on which the claims of the efficacy of HA-1A are based [1] has been the subject of considerable controversy and debate, and the possibility that HA-1A might even have been harmful in some patients has been raised [8–11]. A second clinical study of HA-1A, started in 1992, was suspended in 1993 after interim analysis revealed increased mortality in patients without gram-negative bacteraemia who received HA-1A compared with those who received placebo, and all supplies of HA-1A were recalled [8, 12].

Cytokine production after administration of HA-1A has received little attention, but concern has been expressed on the possible adverse effects of modulation of cytokine expression in certain patients after treatment with HA-1A, in particular an increase in plasma concentrations of IL-6 [13, 14]. Among the cytokines thought to be involved in the systemic inflammatory response to sepsis, plasma concentrations of IL-6 are the most consistently related to outcome in clinical studies, with increased IL-6 concentrations corresponding to an increased risk of mortality [2, 4, 5, 15].

Since HA-1A was withdrawn from clinical use in January 1993, investigation of changes in cytokine concentrations after its use in patients was not possible. Therefore, the effects of HA-1A on cytokine production were studied in vitro, when added to whole blood from ICU patients.

Patients and methods

We studied patients admitted to ICU over a 3-month period. Patients were included if they had SIRS (defined as the presence of two or more of the following: hypo- or hyperthermia; tachycardia; tachypnoea or hypercapnia; leucopenia or leucocytosis according to the ACCP/SCCM Consensus Conference Committee definitions [6]). Inclusion of patients was related to the number of patients in the ICU at any one time (a maximum of three patients were studied simultaneously) and to the ability to process the blood samples. Sampling was performed
mined by radioimmunoassay [17,18] and concentrations of cytokines before and after storage from another 12 ICU patients with SIRS were compared. The effect of incubating blood samples on cytokine production was explored further. Thirty samples in normal subjects was investigated using whole blood from 10 healthy volunteers, using the same procedure 

Because of insufficient specimen volumes, baseline samples were not available in every case. Therefore, the effect of incubating blood samples on cytokine production was explored further. Thirty samples from another 12 ICU patients with SIRS were incubated for 24 h at 37 °C without additives, and concentrations of cytokines before and after storage were compared.

The cytokine response to incubation with HA-1A in normal subjects was investigated using whole blood from 10 healthy volunteers, using the same design as for the ICU patients.

Concentrations of IL-1β and IL-6 were determined by radioimmunoassay [17,18] and concentrations of TNFα by enzyme-linked immunosorbent assay (ELISA) [19]. Lower limits of detection for IL-1β, IL-6 and TNFα were 1.72 ng ml⁻¹, 0.33 ng ml⁻¹ and 7.5 u. ml⁻¹, respectively, while coefficients of variation for the assays were 4.6%, 2.7% and 9.4%, respectively.

Statistical analysis was performed using the Mann-Whitney rank sum test, Wilcoxon signed rank sum test and two-sided Fisher's exact test, as appropriate. Statistical significance was taken as \( P < 0.05 \).

### Results

Fifteen patients were studied (table 1). A total of 61 paired (control and test) samples were analysed, with a median of four paired samples per patient (range 1–20). Seventeen baseline samples were analysed.

Concentrations of IL-6 in controls ranged from undetectable to 3.73 ng ml⁻¹ in all but one patient (No. 6), in whom the initial concentration was 18.76 ng ml⁻¹, decreasing on subsequent days. The median concentration of IL-6 was similar in the 10 survivors (1.35 (range < 0.33–3.73) ng ml⁻¹) and in the five non-survivors (1.67 (< 0.33–18.76) ng ml⁻¹). After incubation with HA-1A, concentrations of IL-6 exceeded control concentrations of IL-6 by more than 25% on 11 occasions in blood from five patients, of whom four died (patients Nos 3, 8, 9 and 11; fig. 1). This compares with one death (patient No. 6) among 10 patients for whom IL-6 concentrations were not greater (table 2). The fifth patient (No. 2) whose blood exhibited greater test concentrations of IL-6 developed severe SIRS after routine admission follow oesophagectomy (no organisms were recovered), and had a protracted stay in the ICU requiring inotropic support before recovering (fig. 2). Patient No. 6, the only patient who died for whom incubation with HA-1A did not result in greater production of IL-6, had very high control concentrations of IL-6, decreasing on subsequent

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Length of stay (days)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48</td>
<td>M</td>
<td>Pneumonia, gram-positive sepsis</td>
<td>14</td>
<td>S</td>
</tr>
<tr>
<td>2</td>
<td>75</td>
<td>F</td>
<td>Post-oesophagectomy, suspected sepsis</td>
<td>28</td>
<td>S</td>
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<td>3</td>
<td>66</td>
<td>F</td>
<td>COAD, chest infection</td>
<td>4</td>
<td>D</td>
</tr>
<tr>
<td>4</td>
<td>84</td>
<td>F</td>
<td>Aspiration of gastric contents after operation</td>
<td>2</td>
<td>S</td>
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<tr>
<td>5</td>
<td>79</td>
<td>F</td>
<td>Routine postoperative admission</td>
<td>1</td>
<td>S</td>
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<tr>
<td>6</td>
<td>79</td>
<td>M</td>
<td>Post-laparotomy for faecal peritonitis</td>
<td>3</td>
<td>D</td>
</tr>
<tr>
<td>7</td>
<td>79</td>
<td>M</td>
<td>Routine postoperative admission</td>
<td>1</td>
<td>S</td>
</tr>
<tr>
<td>8</td>
<td>69</td>
<td>F</td>
<td>Post-laparotomy for intra-abdominal infection, gram-negative bacteraemia</td>
<td>8</td>
<td>D</td>
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<tr>
<td>9</td>
<td>50</td>
<td>F</td>
<td>Cardiac arrest outside hospital from unknown cause, aspiration of gastric contents during resuscitation</td>
<td>2</td>
<td>D</td>
</tr>
<tr>
<td>10</td>
<td>87</td>
<td>F</td>
<td>Post-laparotomy for rectal bleeding</td>
<td>5</td>
<td>S</td>
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<tr>
<td>11</td>
<td>72</td>
<td>M</td>
<td>COAD, chest infection, gram-negative bacteraemia</td>
<td>8</td>
<td>D</td>
</tr>
<tr>
<td>12</td>
<td>73</td>
<td>M</td>
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<td>5</td>
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<tr>
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<td>77</td>
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<td>Routine postoperative admission</td>
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<td>14</td>
<td>51</td>
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<td>Post-laparotomy for bleeding DU</td>
<td>1</td>
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<td>15</td>
<td>71</td>
<td>F</td>
<td>Routine postoperative admission</td>
<td>1</td>
<td>S</td>
</tr>
</tbody>
</table>

### Table 1

Details of patients studied for in vitro effects of HA-1A. Outcome: S = survival, D = death. COAD = Chronic obstructive airways disease, DU = duodenal ulcer. Sepsis was defined as SIRS in the presence of microbiologically proven infection [6].

Both during SIRS and, for those patients who survived, during recovery.

After discussion with the chairman of the Hospital Ethics Committee, informed consent was not obtained from the patients, as the samples used were those already obtained routinely for estimation of C-reactive protein (CRP). Peripheral blood was collected into sterile endotoxin-free tubes containing lithium heparin (Becton Dickinson Ltd, Oxford, UK), which are regularly tested for their inability to induce cytokine production in peripheral blood cells [16]. All subsequent handling of samples was in a hooded cabinet with sterile pyrogen-free equipment, and sterile solutions and reagents. Blood (2 ml) was transferred into each of two sterile endotoxin-free tubes (Flow Laboratories) and 8 μl of HA-1A (Centoxin kindly donated by Centocor, UK) was added to one of the tubes (test) but not to the other (control), after which both tubes were incubated at 37 °C for 24 h. The samples were then centrifuged and the plasma frozen for subsequent analysis of cytokine concentrations. The amount of HA-1A used was that which would produce a similar concentration (20 μg ml⁻¹) in the whole blood sample to that in the peripheral bloodstream after therapeutic infusion [2]. Baseline samples of plasma (i.e. without incubation) were frozen for analysis also. Because of insufficient specimen volumes, baseline samples were not available in every case. Therefore, the effect of incubating blood samples on cytokine production was explored further. Thirty samples from another 12 ICU patients with SIRS were incubated for 24 h at 37 °C without additives, and concentrations of cytokines before and after storage were compared.

The cytokine response to incubation with HA-1A in normal subjects was investigated using whole blood from 10 healthy volunteers, using the same design as for the ICU patients.

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Statistical analysis was performed using the Mann-Whitney rank sum test, Wilcoxon signed rank sum test and two-sided Fisher's exact test, as appropriate. Statistical significance was taken as \( P < 0.05 \).
HA-1A (Centoxin) and cytokine production

Table 2  Outcome in ICU patients according to whether IL-6 concentrations in whole blood incubated with HA-1A exceeded those in whole blood incubated without HA-1A by more than 25%. $P = 0.03$ (Fisher's exact test)

<table>
<thead>
<tr>
<th></th>
<th>Died</th>
<th>Survived</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 &gt; control</td>
<td>4</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>IL-6 &lt; control</td>
<td>1</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>5</td>
<td>10</td>
<td>15</td>
</tr>
</tbody>
</table>

days until his death (fig. 3). There were no differences in median APACHE II scores or white cell count at the time of sampling for patients in whom IL-6 increased (11 (range 6-24) and 13.7 (7.2-31.0) x 10^6 litre$^{-1}$) and for those in whom IL-6 did not increase (10 (3-26) and 9.0 (4.1-22.2) x 10^6 litre$^{-1}$), respectively. Test concentrations of IL-6 which were lower than control concentrations by more than 25% occurred on three occasions in three patients (patient Nos 2, 6 and 8) and were not accompanied by changes in other cytokines.

Control plasma TNFα concentrations were all less than 35 u. ml$^{-1}$ (median 10.0 u. ml$^{-1}$). Concentrations of TNFα in samples incubated with HA-1A could not be determined accurately because of interference of HA-1A with the ELISA for TNFα (subsequently found to be caused by recognition of HA-1A by the anti-mouse antiserum used in the assay [20]). This interference always results in falsely high values of TNFα [20]; thus the median values obtained for TNFα after incubation with HA-1A (18.5 (< 7.5-84.6) u. ml$^{-1}$) were greater than those of the control samples (10.0 (< 7.5-35.2) u. ml$^{-1}$) ($P < 0.001$). However, on those 11 occasions when IL-6 concentrations were increased, median TNFα concentration was similar in control (17.0 (< 7.5-27.4) u. ml$^{-1}$) and HA-1A (18.8 (< 7.5-50.8) u. ml$^{-1}$) samples, despite the interference. It can be concluded therefore that as concentra-
occur had a higher mortality than those in whom it did not occur.

TNFa concentrations did not increase. Further-

patients contained greater concentrations of IL-6

with HA-1A for 24 h, whole blood from some ICU

These results demonstrated that after incubation

not shown).

increases in all three cytokines (IL-1p from 0.46 to

One sample pair from patient No. 8 revealed

from 1.0 to 57000 pg ml-

No. 6 had a higher concentration of IL-6 in whole

No. 6 suffered from gram-positive bacteraemia on occasion A and gram-negative

recovered by worsening in other groups was realized in a second clinical trial, which was halted after interim analysis revealed higher mortality in patients without gram-negative bacteraemia who received HA-1A compared with those who received placebo [8]. Cytokine concentrations in these patients have not been published.

Experimentally, HA-1A does not prevent endotoxin-induced cytokine production in vivo and in vitro [21, 22]. Only one study has examined cytokine concentrations after therapeutic infusion of HA-1A in patients [15]. This was a separate analysis of a subset of 82 patients with suspected gram-negative sepsis, taken from the study by Ziegler and colleagues [1]. Serum concentrations of TNFα were measured in 65 patients and concentrations of IL-6 were measured in 67 patients [15]. Individual variation in serum cytokine concentrations were large: for example, TNFα concentrations ranged from 5.0 to 2264 pg ml⁻¹, and IL-6 concentrations from 1.0 to 57000 pg ml⁻¹. Median decreases in cytokine concentrations were reported at 1 and 24 h after inclusion into the study but no information was supplied on individual changes, in particular if serum IL-6 increased in any patient who received HA-1A. However, the authors did state that IL-6 concentrations increased in 12 of 60 patients who survived for 24 h, of whom eight eventually died. Whether these patients received HA-1A or placebo was not stated. As the drug is no longer available for therapeutic use, this cannot be studied further at present. However, two letters published before withdrawal of HA-1A have drawn attention to the possibility that concentrations of IL-6 may increase in some patients after administration [13, 14], and it is tempting to extrapolate the in vitro findings presented above to these anecdotal reports. Magee, Halliday and Rowlands [13] described eight patients who received HA-1A, of whom three exhibited increases in IL-6 concentrations after treatment (and subsequently died). In contrast, there were no deaths in the five patients in whom IL-6 concentrations decreased after treatment or were normal throughout. TNFα was detected only sporadically [13]. Similarly, Riches, Gooding and Soni [14] reported higher concentrations of IL-6 in two patients who had received HA-1A compared with patients who had not. In addition, concentrations of IL-6 in whole blood from one of these patients increased after incubation for 4 h in vitro with additives, suggesting spontaneous production, whereas no change occurred in whole blood from patients who had not received HA-1A. No increase in TNFα concentrations occurred [14].

HA-1A was introduced for treatment of gram-negative bacteraemia, although many patients presumed to suffer from gram-negative sepsis (i.e. those with SIRS and presumed gram-negative infection [6]) are subsequently found not to have gram-negative infection [1]. Therefore, it seemed appropriate to study the effects of HA-1A in patients

Discussion

These results demonstrated that after incubation with HA-1A for 24 h, whole blood from some ICU patients contained greater concentrations of IL-6 than blood incubated alone, whereas IL-1β and TNFα concentrations did not increase. Furthermore, those patients in whose blood this response occurred had a higher mortality than those in whom it did not occur.
with SIRS but without gram-negative infection, especially when it is this latter group of patients who might be at risk of adverse effects after treatment with HA-1A [8–11].

A possible factor in the present study was contamination with endotoxin. The HA-1A preparation and plasma samples were not tested for endotoxin, so this possibility cannot be excluded definitively. However, contamination is unlikely for several reasons. First, the same procedure, including aseptic precautions, was followed for all 61 pairs of samples, of which only 11 exhibited the IL-6 response; thus contamination of HA-1A is unlikely. Second, the likelihood that chance contamination should have predicted four of five deaths is remote (table 1). Third, in those 11 samples in which IL-6 concentrations were greater than control, concentrations of IL-1β and TNFα were unaffected, whereas all three of these cytokines are produced in response to endotoxin in whole blood [16,23,24]. There are many problems in measuring cytokines in critically ill patients [25]. However, for increases in IL-6 of the magnitude observed, the immunoassays used are sensitive enough to detect accompanying increases in IL-1β and TNFα, after stimulation of whole blood with endotoxin [26]. Contamination is likely to have occurred in the sample from one patient incubated for 24 h in the absence of HA-1A, in which all three cytokines were increased.

These results support the suggestion that peripheral blood from some ICU patients with severe SIRS might be “primed” in terms of cytokine production [27], such that a subsequent stimulus (in this case HA-1A) is able to produce an atypical response (increased IL-6 production); moreover, patients whose blood exhibits this response appear to have increased mortality. The lack of baseline samples for most patients (i.e. not incubated) implies that we cannot be certain that HA-1A actually “induced” production of IL-6, as the starting point of IL-6 concentrations was unknown. For the three patients in whom IL-6 production was increased with HA-1A and for whom baseline samples were available, it would appear that induction of IL-6 by HA-1A did occur. All that can be said for the remaining patients is that IL-6 concentrations were higher after incubation with HA-1A than after incubation without HA-1A. Spontaneous production of cytokines is generally thought not to occur in the absence of contaminating endotoxin, although the above results suggest that this might not always be the case in ICU patients. Furthermore, measurement of plasma cytokine concentrations may be a relatively insensitive method of determining activation of peripheral blood cells [28]. It is possible that circulating endotoxin might activate inflammatory cells and “prime” them for subsequent cytokine production, but small amounts of endotoxin have been shown to suppress, rather than enhance, the response to subsequent endotoxin challenge [29]. Moreover, determination of plasma endotoxin was not attempted in the present study, although the value of this is questionable [7].

The mechanism by which HA-1A might induce production of IL-6 is uncertain, but may involve a direct effect of HA-1A itself, immune complexes containing HA-1A and endotoxin or other bacterial components, HA-1A/HA-1A binding antibody complexes, or a combination of these, on “primed” peripheral blood mononuclear cells (PBMC). After administration of HA-1A to critically ill patients, formation of immune complexes with endotoxin or pre-existing anti-mouse or heterophile antibodies would occur if these were present. Up to 40% of subjects have significant amounts of multivalent antibodies in their plasma that are able to bind to mouse IgG [30]; the ability of these antibodies to bind to HA-1A has not been assessed, although this possibility is suggested by the finding that anti-mouse IgG antiserum does bind to HA-1A experimentally [20]. Formation of larger complexes is possible if endotoxin is involved also. It is possible that formation of immune complexes represents one mechanism whereby HA-1A affects cytokine production in whole blood from some patients in the ICU, and that similar mechanisms are responsible for adverse effects after therapeutic administration [8,13,14].

The lower test concentrations of IL-6 compared with controls on three occasions raises the possibility that either HA-1A might have increased clearance or breakdown of IL-6, or that spontaneous production might occur in whole blood from some patients, and that HA-1A might prevent this. There is no evidence to suggest binding of HA-1A to IL-6 or enhanced breakdown of IL-6; indeed if this were the case one would have expected a similar phenomenon to be more consistent than is found in the present study. The question of spontaneous production of cytokines ex vivo has received attention previously. Early reports of spontaneous cytokine production in blood samples have since been attributed to contaminating endotoxin, and it has been demonstrated that in the absence of such contamination, production of IL-1, IL-6 and TNFα is not induced in whole blood from healthy volunteers [16,31]. However, the situation in critically ill patients may be different. Riches, Gooding and Millar reported that when blood samples from seven such patients were analysed, concentrations of both IL-1β and IL-6 were stable for 3 h over a wide range of initial values [16]. However, a recent study reported spontaneous production of IL-6 by isolated PBMC from patients with pancreatic cancer [32]. In the present study, spontaneous production of cytokines occurred in some cases. Although contamination with endotoxin cannot be ruled out, this is unlikely in view of the lack of IL-1β and TNFα response. It is feasible that in the presence of a circulating stimulus, slow spontaneous production of cytokines may indeed occur only in a few patients. For HA-1A to prevent this production, as in the three patients presented here (patient Nos 2, 6 and 8), one can only speculate that it might do so by binding to endotoxin, endotoxin-containing immune complexes or HA-1A-binding antibodies. However, it is not known if circulating endotoxin was present in these patients. Only one patient (No. 8) had proven gram-negative bacteraemia, but this may occur in the absence of detectable endotoxaemia [7,15]. In addition, endo-
toxaemia has been reported in patients without gram-negative infection, including those infected with gram-positive organisms [7,15]. Indeed, the spontaneous production of IL-6 that was demonstrated in vitro was associated with proven gram-positive bacteraemia on at least two out of the three occasions. Moreover, the lack of IL-1β and TNFα production in vitro would argue against circulating endotoxin causing spontaneous secretion of IL-6 alone, unless down-regulation of only the former cytokines had occurred; whether or not this may occur clinically is uncertain.

In such a complex setting as SIRS and sepsis, where regulatory mechanisms are being stressed severely, it is perhaps naïve to suppose that modulation at a single point in the inflammatory pathway might be able to correct the homeostatic imbalance. This would be especially pertinent to the use of HA-1A and other anti-endotoxin therapies, as even the role of endotoxin itself is disputed in these conditions [7]. The above data suggest caution in the introduction of immunotherapies aimed at disrupting the cytokine cascade, without thorough prior investigation of their effects on cytokine production.

Acknowledgements

S.M.Y. was supported by Smiths Industries Medical Systems.

References

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