Cytokine Patterns in Patients After Major Vascular Surgery, Hemorrhagic Shock, and Severe Blunt Trauma
Relation with Subsequent Adult Respiratory Distress Syndrome and Multiple Organ Failure

Rudi M. H. Roumen, M.D., Ph.D.,* Thijs Hendriks, Ph.D.,* Johanna van der Ven-Jongekrijg,†
Grard A. P. Nieuwenhuijzen, M.D.,* Robert W. Sauerwein, M.D., Ph.D.,‡
Jos W. M. van der Meer, M.D., Ph.D.,† and R. Jan A. Goris, M.D., Ph.D.*

From the Departments of Surgery,* Internal Medicine,† and Microbiology,‡ University Hospital
Nijmegen, Nijmegen, The Netherlands

Objective
This study investigates the course of serum cytokine levels in patients with multiple trauma,
patients with a ruptured abdominal aortic aneurysm (AAA), and patients undergoing elective AAA
repair and the relationship of these cytokines to the development of adult respiratory distress
syndrome (ARDS) and multiple organ failure (MOF).

Summary Background Data
Severe tissue trauma, hemorrhagic shock, and ischemia–reperfusion injury are pathophysiologic
mechanisms that may result in an excessive uncontrolled activation of inflammatory cells and
mediators. This inflammatory response is thought to play a key role in the development of
(remote) cell and organ dysfunction, which is the basis of ARDS and MOF.

Methods
The study concerns 28 patients with multiple trauma, 20 patients admitted in shock because of a
ruptured AAA, and 18 patients undergoing elective AAA repair. Arterial blood was serially sampled
from admission (or at the start of elective operation) to day 13 in the intensive care unit, and the
serum concentrations of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 were
determined.

Results
Twenty-two patients died, 15 within 48 hours and 7 after several weeks, as a result of ARDS/MOF.
At hospital admission and after 6 hours, these nonsurvivors had significantly higher plasma TNF-α
and IL-1β levels than did the survivors. At the same measuring points, TNF-α and IL-1β were
significantly more elevated in patients with ruptured AAA than in traumatized patients. However,
IL-6 was significantly higher in the traumatized patients. In 10 patients, ARDS/MOF developed,
and 41 had an uncomplicated course in this respect. Those with ARDS/MOF exhibited
significantly different cytokine patterns in the early postinjury phase. TNF-α and IL-1β levels were
higher mainly on the first day of admission; IL-6 concentrations were significantly elevated in
Evidence is accumulating that adult respiratory distress syndrome (ARDS) and multiple organ failure (MOF) are the result of a severe generalized autodestructive inflammation, in which microorganisms may or may not be involved. Trauma, shock, and infection initiate a complex inflammatory response, in which the proinflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 are thought to play a pivotal role. The role of these cytokines has almost exclusively been studied in relation to the pathogenesis of infection and septic shock. Only a few studies deal with cytokinemia after trauma or hemorrhagic shock. Most of these—clinical and experimental—studies have death as their end point. However, ARDS and MOF represent an important part of the morbidity incidence in the intensive care unit (ICU), being responsible for more than 70% of the ventilator days spent on the ICU. In addition, ARDS and MOF are the main cause of late death in surgical ICU patients and put a heavy burden on health care. Therefore, unraveling the pathophysiology of these syndromes should be a major objective of study. We investigated the role of circulating cytokines (TNF-α, IL-1β, and IL-6) in relation to the development of ARDS and MOF in patients after essentially nonbacterial challenges, that is, severe blunt trauma, hemorrhagic shock, and major vascular surgery.

PATIENTS AND METHODS

Sixty-six patients were studied prospectively, including 18 patients undergoing elective aortic surgery (16 with abdominal aortic aneurysms [AAA] and 2 with thoracoabdominal aortic aneurysms), 20 patients with hemorrhagic shock because of a ruptured AAA, and 28 patients with severe blunt trauma (hospital trauma index injury severity score [ISS] > 25). All patients or their relatives gave informed consent to draw blood for the analyses.

Arterial blood was withdrawn from the patients undergoing elective aortic surgery at the time of the incision, when they had already been anesthetized (0 hours) and after 6 and 24 hours. Subsequently, samples were collected daily during the first week and every other day during the second week, as long as the patients were in the ICU, with a maximum of 2 weeks. For the other patients, the first sampling point (0 hours) was immediately after hospital admission. Thereafter, the same schedule was followed as for the patients with elective surgery.

Conclusions

In the early postinjury phase, higher concentrations of these cytokines are associated, not only with an increased mortality rate, but also with an increased risk for subsequent ARDS and MOF. These data therefore support the concept that these syndromes are caused by an overwhelming autodestructive inflammatory response.

Definitions

An APACHE II score was calculated for all patients at the time of hospital and subsequent ICU admission and, thereafter, daily throughout the ICU stay. To grade the intensity of organ failure, a daily MOF score was calculated. The patient's condition was diagnosed as MOF if the average MOF score between days 5 and 14 was ≥ 4. ARDS was diagnosed when patients had bilateral diffuse infiltrates on chest radiography and progressive hypoxemia requiring mechanical ventilation, resulting in an arterial oxygen tension–fraction of inspired oxygen ratio ≤ 175 with PEEP ≥ 10 cm H₂O and, in cases of prior cardiac disease, did not have a pulmonary artery wedge pressure exceeding 18 mmHg. A shock score was defined to grade the severity of hemodynamic derangement after admission. For this purpose, the Allgöwer shock index (heart frequency/systolic blood pressure) and the systolic blood pressure were used as follows: 0 or no shock, index ≤ 1.0 and systolic blood pressure ≥ 100 mmHg; 1 or mild, compensated shock, index > 1.0 and systolic blood pressure ≥ 100 mmHg; 2 or moderate shock, systolic blood pressure 80 to 100 mmHg; 3 or severe shock, systolic blood pressure < 80 mmHg; 4 or severe, prolonged shock, more than 1-hour systolic blood pressure < 80 mmHg.

Blood Analysis

Arterial blood was collected in sterile 4-mL tubes containing 0.048 mL of ethylene diamine tetra-acetate (Vacutainer Systems, Becton Dickinson, Rutherford, NJ) and 250 μL of aprotinin (final concentration, 625 kallikrein inactivating units/mL, Bayer, Leverkusen, Ger-

Send correspondence to R. M. H. Roumen, M.D., Ph.D., Department of Surgery, University Hospital Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands. Accepted for publication April 21, 1993.
Cytokines After Injury and Hemorrhagic Shock

many). After centrifugation at 2000 \( \times g \) for 10 minutes, the resulting platelet poor plasma was transferred into sterile 1.5-mL tubes, and the samples were stored at -20°C until further analysis. The samples were analyzed by fluid-phase radioimmunoassay for TNF-\( \alpha \) and IL-1\( \beta \), as described previously.\(^{19,20} \) Before the IL-1\( \beta \) measurement, the plasma was extracted with chloroform according to a published method.\(^{20} \) The detection levels in the assays were as follows: TNF-\( \alpha \), 60 pg/mL; IL-1\( \beta \), 200 pg/mL.

Arterial blood for the IL-6 assay was collected in sterile 4-mL tubes and processed as described earlier. IL-6 was quantified by an enzyme-linked immunosorbent assay. Microtiter flat-bottom plates (Costar, The Netherlands) were coated with anti-IL-6 monoclonal (Moab) BE-8 (7 \( \mu g/mL \)) in phosphate-buffered saline (PBS), pH 7.4 (100 \( \mu L/well \)). After a 24-hour incubation at 4°C, the plates were washed with PBS with 0.02% (v/v) Tween-20, followed by a 60-minute room temperature incubation of PBS with 5% bovine serum albumen (BSA) (200 \( \mu L/well \)). After washing, serial dilutions of standard recombinant human IL-6 (a kind gift of Dr. L. Aarden, CLB, Amsterdam, The Netherlands) or the serum samples were added to the plates, and incubation proceeded for 1 hour at 37°C. After washing, 100 \( \mu L \) of PBS/BSA with biotinylated anti-IL-6 Moab BE-4 (2 \( \mu g/mL \)) was added to the wells. BE-4 and BE-8 recognize different epitopes. After a 60-minute incubation and a washing step, 100 \( \mu L \) of strepavidin horseradish peroxidase in PBS/BSA was added, followed by a 45-minute incubation. After washing, a citric acid buffer, pH 5.2, with 0.003% hydrogen peroxide and ortho-phenyldiamine was added. The reaction was terminated with 4 N sulfuric acid, and the absorbance was read at 495 nm in an automated enzyme-linked immunosorbent assay reader (Titterek, The Netherlands). The detection level of this assay was 20 pg/mL.

Arterial blood lactate was measured in deproteinized samples (0.6 N perchloric acid) by enzymatic conversion. The detection level of this assay was 20 pg/mL.

Table 1. We differentiated between early (the first or second day of admission) and late death. Fifteen patients (eight with trauma and seven with ruptured AAAs) died early. Of the remaining 51 patients, in 10, ARDS/MOF developed. Two traumatized patients had ARDS without subsequent MOF and survived. One patient with a ruptured AAA had ARDS without MOF and also survived. The remaining seven patients all had MOF (six including ARDS), and they died on days 28 to 60 in the ICU. Thus, the total number of deaths was 22 in 66 patients (33%). The 41 survivors who did not have ARDS/MOF were considered to be patients with an uncomplicated clinical course.

The course of the cytokine concentrations during the first 2 days in the three patient groups is depicted in Figure 1. On admission, IL-1\( \beta \) and, after 6 hours, TNF-\( \alpha \) levels were at a significantly higher level in patients with ruptured AAAs compared with the traumatized patients (\( p = 0.03 \) and 0.02, respectively). In this early postinjury period (0 hours), however, the IL-6 level was significantly more elevated in the traumatized patients compared with both vascular patient groups (\( p < 0.001 \)). On days 1 and 2, the differences between groups in regard to TNF-\( \alpha \) and IL-1\( \beta \) levels disappeared, but IL-6 concentrations were increased in the vascular patient groups. In the traumatized patients, a decrease was observed.

Figure 2 illustrates the course of cytokine concentrations during the first day of admission in relation to death. The corresponding \( p \) values of these measurements are listed in table 2. Nonsurvivors had significantly higher TNF-\( \alpha \) and IL-1\( \beta \) concentrations, both on admission and 6 hours later. With respect to IL-1\( \beta \), this difference was mainly caused by the contribution of the late nonsurvivors who died of ARDS/MOF; in the case of TNF-\( \alpha \), both early and late nonsurvivors appeared to contribute equally to the observed difference with survivors. The differences in IL-6 concentrations between nonsurvivors and survivors did not reach statistical significance (\( p = 0.07 \) and 0.08 at 6 and 24 hours, respectively). In addition, we did not find any significant difference in cytokine concentrations between early and late nonsurvivors.

Figure 3 shows the course of the cytokine concentrations during the first 2 weeks in the ICU in the group of 10 patients with ARDS/MOF and the 41 patients with an uncomplicated course. The IL-1\( \beta \) level was increased in patients with ARDS/MOF at 6 hours and at the end of day 1, but the difference did not reach statistical significance (\( p = 0.056 \) and 0.087, respectively). This difference completely disappeared between days 2 and 4 because the uncomplicated patients had a rise in IL-1\( \beta \) concentrations. In the subsequent period, IL-1\( \beta \) levels decreased again in the uncomplicated group, but the levels remained elevated in the patients with ARDS/MOF.

TNF-\( \alpha \) showed a somewhat similar picture. There was a significant difference in TNF-\( \alpha \) levels at 6 hours (\( p < 0.005 \)) and an almost significant difference between days

Statistical Analysis

The Kruskal–Wallis test and the Wilcoxon two-sample test were used to examine differences between patient groups when indicated. Spearman’s correlation coefficients were used for correlation analysis. We considered \( p \) values < 0.05 to be significant.

RESULTS

Demographic data for the 66 patients are presented in Table 1. We differentiated between early (the first or second day of admission) and late death. Fifteen patients (eight with trauma and seven with ruptured AAAs) died

Cytokines After Injury and Hemorrhagic Shock

771
Table 1. DEMOGRAPHIC DATA OF THE STUDY POPULATION

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Age (yr)</th>
<th>Sex (F/M)</th>
<th>APACHE II on ICU Admission</th>
<th>Injury Severity Score</th>
<th>Early Death (&lt;48 hr)</th>
<th>ARDS and/or MOF</th>
<th>Late Death (&gt;48 hr)</th>
<th>Uncomplicated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elective</td>
<td>18</td>
<td>64 ± 11</td>
<td>3/15</td>
<td>6.2 ± 4.0</td>
<td>—</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>Ruptured</td>
<td>20</td>
<td>70 ± 7</td>
<td>1/19</td>
<td>11.6 ± 5.4</td>
<td>—</td>
<td>7</td>
<td>5</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Trauma</td>
<td>28</td>
<td>31 ± 14</td>
<td>6/22</td>
<td>9.9 ± 7.0</td>
<td>38 ± 11</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>Total</td>
<td>66</td>
<td>10/56</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MOF = multiple organ failure.
Values are mean ± SD.

1 and 2 (p = 0.055 and 0.051, respectively). During the second week, TNF-α levels rose in patients with ARDS/MOF and tended to normalize in the uncomplicated patient group.

The IL-6 levels followed a completely different course. At all measuring points, the IL-6 concentrations were significantly higher in the patients with ARDS/MOF than in the uncomplicated patients, except for the first 24 hours, because of their large variations.

A good correlation was found between the ISS score and IL-6 levels on admission and 6 and 24 hours later (r = 0.52, 0.43, and 0.53, respectively; all p < 0.05). A highly significant correlation (r = 0.83, p < 0.001) was found between IL-6 levels and lactate concentrations at 24 hours. Further correlation analysis between cytokines and the ISS score, shock score, lactate concentrations, or APACHE II score did not show any consistent pattern of significant correlations.

In all patients, a daily MOF score was calculated, and we correlated these values with the cytokine levels measured at different days. During the second week, IL-1β and TNF-α showed good correlations with the MOF score (e.g., IL-1β levels on day 9 and MOF scores on day 9 [r = 0.63, p = 0.02]; IL-1β levels on day 11 and MOF score on day 13 [r = 0.68, p = 0.02]; TNF-α concentrations on day 7 and MOF score on day 7 [r = 0.77, p = 0.005]; and TNF-α levels on day 9 and MOF score on day 11 [r = 0.84, p = 0.001]).

Finally, striking correlations (all p < 0.01) were found between IL-6 concentrations and MOF scores from the second day onward (e.g., day 2, r = 0.64; day 3, r = 0.53; day 4, r = 0.60; day 5, r = 0.77; day 7, r = 0.77; day 9, r = 0.59; and day 11, r = 0.63).

Correlation analysis between the cytokine concentrations of all samples at various time points did not reveal any significant correlation.

**DISCUSSION**

In the present study, we found intriguing differences in cytokine profiles between three different patient groups representing different pathogenic conditions associated with an increased risk for subsequent ARDS/MOF, that is, severe trauma, hemorrhagic shock (ruptured AAA), and ischemia–reperfusion injury (elective AAA). On admission and after 6 hours, TNF-α concentrations were significantly higher in patients after hemorrhagic shock than after trauma. In the former patients, higher IL-1β concentrations were also found on admission. These findings are in accordance with animal experiments showing that the induction of TNF-α release after trauma is significantly enhanced when hemorrhagic shock is also present. Apparently, shock is the main factor leading to the production and release of TNF-α by macrophages. Experimentally, it has also been demonstrated that intestinal ischemia and reperfusion injury leads to five- to tenfold increases of circulating TNF-α levels and that hypoxia alone is a major stimulus for human peripheral blood monocytes to produce and secrete IL-1β and TNF-α.

The ischemia and reperfusion injury is likely to be responsible for the elevated levels of TNF-α and IL-1β found in patients after elective aortic aneurysm repair. However, it is unclear why, in some of these patients after induction of anesthesia and at the start of the operation, increased TNF-α and IL-1β concentrations were found. The use of specific medication, such as cyclooxygenase inhibitors, or the presence of chronic heart failure has been shown to lead to raised levels of circulating TNF-α. However, only one patient (TNF-α < 60 pg/mL) regularly received cyclooxygenase inhibitors; one other patient exhibited signs of mild chronic heart failure (TNF-α, 110 pg/mL). Certainly, the role of anesthesia in relation to the release of these cytokines needs further investigation.

Our observations of increased IL-1β concentrations after major trauma are in agreement with the findings of another group, who demonstrated short-lived IL-1β responses to major surgical injury. It is more difficult to explain how our data relate to those of others, who showed that the elevated number of circulating mono-
cytes found postinjury were not able to produce adequate amounts of IL-1β within the first 8 to 10 days after trauma. This process was attributed to elevated concentrations of prostaglandin E₂, which downregulated TNF-α and IL-1β synthesis. However, it is currently unclear to what extent circulating monocytes contribute to the in vivo production of cytokines. TNF-α and IL-6, but not IL-1β, activity could only be detected in a rat model of major surgery and shock. An explanation could be that we used chloroform extraction to show IL-1β activity. In addition, there may be differences between the cytokine responses in rats and humans.

We found a different profile for IL-6. Traumatized patients exhibited the highest concentrations of IL-6 directly postinjury and 6 hours later. These data support the idea that IL-6 production is more closely tied to soft tissue trauma than is that of other cytokines. The IL-6 profiles observed are also in agreement with the reports describing a relationship between postoperative conce-
Table 2. P VALUES OF WILCOXON TESTS FOR ANALYSIS OF DIFFERENCES BETWEEN CYTOKINE CONCENTRATIONS OF SURVIVORS AND NONSURVIVORS (INCLUDING EARLY AND LATE NONSURVIVORS)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>All Nonsurvivors</th>
<th>Early Nonsurvivors (n = 15)</th>
<th>Late Nonsurvivors (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.04*</td>
<td>0.32</td>
<td>0.007†</td>
</tr>
<tr>
<td>6</td>
<td>0.001†</td>
<td>0.26</td>
<td>0.004†</td>
</tr>
<tr>
<td>24</td>
<td>0.02*</td>
<td>0.15</td>
<td>0.10</td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.003†</td>
<td>0.01*</td>
<td>0.08</td>
</tr>
<tr>
<td>6</td>
<td>0.002†</td>
<td>0.06</td>
<td>0.01*</td>
</tr>
<tr>
<td>24</td>
<td>0.67</td>
<td>0.12</td>
<td>0.15</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.75</td>
<td>0.44</td>
<td>0.17</td>
</tr>
<tr>
<td>6</td>
<td>0.07</td>
<td>0.006†</td>
<td>0.86</td>
</tr>
<tr>
<td>24</td>
<td>0.08</td>
<td>0.18</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Corresponding cytokine concentrations are demonstrated in Figure 2.

* p < 0.05
† p < 0.01.

The delay in the IL-6 peak in the patients with ruptured and elective AAAs might be explained by the fact that, in this case, IL-6 was mainly released by the surgical trauma of the vascular reconstruction and IL-6 levels tend to follow TNF-α and IL-1β peaks after some delay.26,27 It is of interest that the occurrence of shock apparently did not contribute significantly to the release of IL-6 because we found no difference between patients after acute and elective major vascular surgery.

The role of translocating bacterial endotoxin in the production and release of the cytokines in the present patient population remains unclarified. In 28 individuals (11 with trauma, 10 with ruptured AAAs, and 7 with electively treated AAAs), we were able to look for blood endotoxin concentrations at 0, 6, 24, and 48 hours.16,28 In 15 patients (4 with trauma, 6 with ruptured AAAs, and 5 with electively treated AAAs), endotoxemia could be detected (mostly at 0 or 6 hours), and all but two patients exhibited circulating TNF-α levels at the same time (data not shown). However, in 10 of the 13 patients without detectable circulating endotoxin levels, we also found increased TNF-α concentrations at one or more of the same time points. Thus, endotoxemia may be indicative of the presence of subsequent circulating TNF-α, but it surely is not a prerequisite. Endotoxemia has been shown to occur after hemorrhagic shock, intestinal ischemia, and reperfusion injury and even after elective aortic aneurysm repair,16,21,28-30 but it was only rarely detected in patients with multiple trauma.31

There is ample evidence that endotoxin induces the production and secretion of TNF-α, but it is clear that endotoxin is not the only factor responsible for the cytokine production in our patients. Various microbial and nonmicrobial factors are known to cause cytokine production.

The present data demonstrate that patients who subsequently die eventually exhibit much higher levels of TNF-α, IL-1β, and IL-6 in the early post-trauma phase than do survivors, but the finding of increased cytokine levels in nonsurvivors was probably not an independent phenomenon. The fact is that the large standard devia-
tions indicate that the finding of elevated cytokine concentrations can hardly be expected to predict the prognosis better in an individual patient than, for example, the ISS or APACHE II score would do. Nevertheless, the present data may help to elucidate the mechanism of inflammatory response to trauma and shock.

Finally, our data demonstrated a good correlation between cytokine levels and the development of ARDS and MOF. On the first 2 days after admission, TNF-α concentrations were significantly elevated in patients with subsequent ARDS/MOF. This finding was in agreement with those of previous reports, in which TNF-α levels were found to correlate with subsequent sepsis or septic episodes in patients postinjury. From these studies, however, it is not clear whether this phenomenon was linked to bacterial or nonbacterial sepsis because the presence of bacteria was not obligatory for the definition of sepsis in these studies. Such obscure use of the term sepsis and septic syndrome has already led to a lot of confusion in the discussion about the relationship between bacterial sepsis (infection) and MOF. It would therefore be better to use a term like systemic inflammatory response syndrome as was recently proposed.

With respect to the IL-1β levels, there only was an indication of a significant elevation in patients with subsequent ARDS/MOF on the first day after admission. An interesting finding was the rise of chloroform-extractable IL-1β between days 2 and 4 in the patients with an uncomplicated clinical course. A similar phenomenon was observed during the recovery phase in patients with meningococcal infections. This finding might suggest a protective effect of IL-1β that is necessary during the course of reconvalescence, which supports the proposition that several cytokines not only are synergistic but also counterregulate each other. However, this observation might also be influenced by the assay method used, and therefore, this phenomenon is presently being investigated further. Patients with ARDS/MOF, on the other hand, showed a trend of sustained elevated TNF-α and IL-1β levels during the second week. This protracted cytokine release was probably associated with the organ damage observed in those patients.

The presence of significantly increased IL-6 concentrations in the patients with subsequent ARDS/MOF was remarkable. This was also reflected by the striking correlation between the IL-6 level and the MOF score during the 2 weeks studied. Although the patients with an uncomplicated clinical course showed a normalization of IL-6 in the second week, the release of IL-6 in the patients with ARDS/MOF remained abnormally high during this period. The same has been demonstrated in patients after major thermal injury and septic shock.

We could not demonstrate any relevant correlation between the concentrations of the three cytokines. This finding has also been reported previously, and therefore, our data support the concept that the production of these cytokines is regulated independently.

In conclusion, after major trauma, hemorrhagic shock caused by a ruptured AAA, or elective aortic aneurysm repair, increased concentrations of the cytokines TNF-α, IL-1β, and IL-6 are a common finding. The higher concentrations found in the early postinjury time course are associated, not only with increased mortality rates, but also with an increased risk for the development of ARDS and MOF. The present study therefore supports the concept that ARDS and MOF are syndromes caused by an excessive uncontrolled activation of endogenous inflammatory cells and mediators.

Acknowledgments

The authors thank K. Huyben and W. Kraak for their excellent technical assistance.

References


