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Risk Assessment for Intensive Care Patients by Automated Classification of Flow Cytometric Data

Günter K. Valet
Max-Planck-Institut für Biochemie, Martinsried, Germany

Gabriel Roth
Institut für Anästhesiologie, Klinikum Grosshadern der Universität, Munich, Germany

Wolfgang Kellermann
Institut für Anästhesiologie, Klinikum Grosshadern der Universität, Munich, Germany

SUMMARY

The early recognition of endangered sepsis patients in intensive care medicine is of great clinical importance. The risk assessment by clinical chemistry or by other clinical parameter does not, however provide this information at an early stage. It was therefore investigated whether metabolic parameters of blood leukocytes such as oxidative burst activity of monocytes and granulocytes as well as leukocyte proteinase activities in conjunction with data pattern classification are more informative.

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The oxidative burst activity in vital leukocyte preparations following 1g sedimentation of fresh heparinized blood was determined with dihydrorhodamine 123 (DHR 123). Furthermore the serine and cathepsin B,H,L proteinase activities were measured using fluorogenic $(Z\text{-}AlaAla)_2$-rhodamine 110 (R 110) and $(Z\text{-}ArgArg)_2$R 110 substrates.

The differences between septic and nonseptic patients were significant and septic patients could be correctly diagnosed in 100% of the cases by automated evaluation of the DHR 123, $(Z\text{-}AlaAla)_2$ R 110, and $(Z\text{-}ArgArg)_2$ R 110 assays with the self-learning data pattern classifier CLASSIF1.

The clinically more important risk assessment of septic patients at admission was equally possible by the DHR 123 and $(Z\text{-}AlaAla)_2$ R 110 assays with a slight advantage for DHR 123. The findings emphasize the potential for early recognition of endangered patients. Nonsurviving patients died between 5 and 14 days after admission to the intensive care unit. The systematic optimization of the classification indicates that the TNF-α- and fMLP-stimulated DHR 123 assays were most informative whereas the serine proteinase, DHR 123 ex vivo, TNF-α + fMLP, and PMA assays contained redundant information that did not further improve the overall classification results.

INTRODUCTION

The management of preseptic or septic patients in intensive care units constitutes an important challenge for the treating physician. Although the determination of sepsis scores as well as biophysical and biochemical measurements of constituents or functions of humoral body compartments such as blood, blood plasma, or urine provides valuable diagnostic information, they are of comparatively low value for a patient's risk assessment, that is, for prognostic purposes.

As a conceptual alternative, the investigation of biochemically specific features of blood cell functions by flow cytometry is of great interest for the individual patient's risk assessment. Disease processes derive from biochemical deviations in cellular system or organs. Once recognized at the cellular level, immediate therapeutic intervention may considerably lower the degree of intracorporeal destruction. This effect increases the chances for fast and complete patient recovery.

Granulocytes are particularly important in sepsis and shock events because of their capacity for phagocytosis and destruction of microorganisms as well as for unbalancing the regulation of the microvasculatory system by the overshooting liberation of enzymes or mediators and by the exposure of new antigens. Some of these antigens may provide increased interaction with the endothelial cell layer.

Early multiparametric determinations of granulocyte parameters by flow cytometry permitted the prediction of the danger of sepsis or noninfectious shock in intensive care patients 2–3 days in advance (Rothe et al., 1993c), that is, at a time when humoral or other patient parameters are entirely unable to indicate the imminent danger.

The measurement of phagocytosis of live *E. coli* by simultaneous acridine orange/propidium iodide (AO/PI) staining (Rothe and Valet, 1988) as well as of the intracellular pH values and esterase activities by 1,4-diacetoxy-2,3-dicyanobenzene/propidium iodide (ADB/PI) staining (Valet et al., 1981) needs no centrifugation steps; that is, such assays are suitable for automated flow cytometric instrumentation.

The handling of live bacteria, however, requires special care and a separate 37°C incubation step prior to fluorescent cell staining. Furthermore, the UV-excitation of the ADB dye is not available in most of the current analytical flow cytometers, and the learning process with statistical classifiers (Rothe et al., 1990) takes substantial time. In addition, statistical classifiers have a tendency to be unstable when comparatively few informative parameters are buried in a majority of "noise," that is, noninformative parameters. The resulting classifiers are not standardized; that is, they are not easily portable.

To overcome these problems, the goals of subsequent work were fourfold:

1. Development of the 488-nm exciting and sensitive oxidative burst indicator dye dihydrorhodamine 123 (DHR 123) (Rothe and Valet, 1993); DHR 123 has become a widespread fluorogenic dye in research on neutrophils (Werusch et al., 1996a,b; Löhrke et al., 1996; Woodman et al., 1995), in general cell biology (Sobreira et al., 1966; Wang et al., 1996; Wersto et al., 1996), and in immunology (Williams and Henkart, 1996), but also in investigations on the formation and action of intracellular peroxinitrite (Zinarelli et al., 1997; Kooy and Lewis, 1996; Briviba et al., 1996).
2. Development of rhodamine 110 (R 110) proteinase substrates for the specific determination of intracellular proteolytic activities in viable cells (Rothe and Valet, 1993a,b; Ganesh et al., 1995; Klingal et al., 1994).
3. Differential exposure of blood leukocytes to humoral stimulators such as bacterial peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), tumor necrosis factor-alpha (TNF-α) and phorbol myristate acetate (PMA) as a positive control instead of stimulation by bacteria.
4. Use of triple matrix classification (Valet et al., 1993) with its capacity to provide instrument- and laboratory-independent, that is, standardized classifiers for routine classification processes in hospitals.

Using these new tools, substantial improvements can be obtained. This is exemplified in the measurement and prospective classification of a series of patients admitted to the intensive care unit either without or with infection of various degree including sepsis. The question of the present work was to what extent the flow cytometrically determined DHR 123 burst and R 110 protease activity measurements were useful for infection diagnosis as well as for predictions on ultimate survivors and nonsurvivors in sepsis.

MATERIALS AND METHODS

Patients

Patients were admitted noninfected, infected, or in the state of sepsis to the anesthesia intensive care unit following general abdominal, orthopedic, polytraumatic or gynecologic surgery. Sepsis was defined as recommended by the ACCP + SCCM consensus conference (American College of Chest Physicians, 1992). The CLASSIF1

classified patients were unselected; that is, the data of all patients in this series were used, with the exception of the data of one survivor and one nonsurvivor patient. Both patients were selected prior to the learning process and constituted a small test set, unknown to previously learned classifiers.

All patients were treated according to current principles of intensive care medicine such as maintenance of sufficient tissue oxygenation, cardiac output, electrolyte balance, and nutrition status, as well as antibiotic therapy, search and removal of septic foci, and so on.

Blood Samples and Cell Staining

Buffy coat leukocytes were obtained following 40-min sedimentation of 2-ml heparinized blood samples (10 IU/ml) at room temperature after overlayering them at a 1:1 volume ratio onto 1.077 g/ml lymphocyte separation medium (Sigma, Deisenhofen, Germany) in polycarbonate test tubes.

Leukocyte samples were diluted 1/50 in 5 m*M* Hepes (Sigma) buffered saline (HBS) pH 7.35 and incubated for 5 min at 37°C in the presence of 1 μ*M* DHR 123 (Molecular Probes, Eugene, Oregon). They were further incubated for 20 min either unstimulated or in separate assays in the presence of physiological stimulators such as 10 ng/ml TNF-α (Sigma, recombinant in yeast 2×10^7 U/mg protein), 100 n*M* fMLP (Sigma), or a combination of both as well as with 100 n*M* PMA (Sigma) as a positive control (Rothe and Valet, 1993c).

Leukocyte samples were further incubated without stimulation in 1/50 HBS dilution for 30 min at 37°C in the presence of 4 μ*M* of either $(Z\text{-}AlaAla)_2$-R 110 serine proteinase or $(Z\text{-}ArgArg)_2$-R 110 cathepsin B,H,L substrate. The substrates were produced by synthesis in our laboratory (Rothe et al., 1992).

Dead cells in all assays were counterstained for 5 min at room temperature with 60 μ*M* propidium iodide (PI, Serva, Heidelberg, Germany).

Flow Cytometry

The stained leukocytes were analyzed in a FACScan flow cytometer (Becton Dickinson, Heidelberg) with linear amplification for sideward light scatter (SSC) and four-decade logarithmic amplification for forward light scatter (FSC) as well as in the FITC (F1) and PI (F3) fluorescence channels. 10,000 cells were collected with Consort30 software as FCS1.0 list mode files. The resulting data files were transferred by the Fastlink program (Becton Dickinson) to a personal computer for further analysis. Day-to-day precision of the flow cytometer was assured by repeated measurements of the same lot of monosized 4.5-μm YG FITC-like calibration particles (Polysciences, Eppelheim, Germany).

List Mode Data Analysis

List mode data were analyzed by the CLASSIF1 program (Partec, Münster, Germany) in a standardized way.

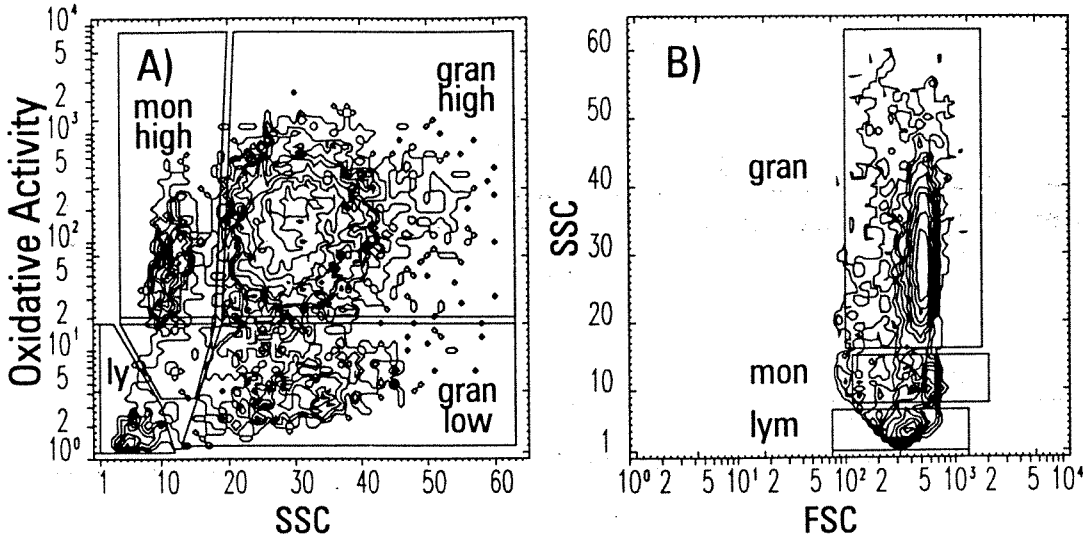

Fig. 1. Standardized evaluation of the DHR 123 oxidative burst measurement of a PMA-stimulated leukocyte sample from a healthy person. The evaluation windows were fixed (A), that is, identical for all list mode files of the learning and test sets to assure the unbiased evaluation. The gates in the light scatter histogram (B) were autogated according to following procedure: the lymphocyte evaluation window is used as leader gate. The evaluation program detects the maximum of the lymphocyte cluster and sets thresholds in predetermined distances to the peak in the *x* and *y* directions (up/down/left/right). The monocyte gate is localized immediately on top of the lymphocyte gate with initially 1.2 times the *y*-span of the lymphocyte gate. The maximum of the monocyte peak is determined and thresholds are set in predetermined distances of the peak. If no monocyte peak is detected, the initial default gate is used. The granulocyte gate is finally placed immediately above the monocyte gate. It spans until the highest *y*-axis channel and has predetermined outreaches to the left and right of the maximum of the granulocyte cluster on the *x*-axis.

Vital and dead cells were discriminated in the F1/F3 histograms (not shown), and the DHR 123 (Fig. 1) and R 110 (Fig. 2) stain of vital lymphocytes, monocytes, and granulocytes were evaluated within the indicated windows. Suitable but fixed evaluation windows for the SSC/FITC histograms and self adjusting gates for the separation of lymphocytes, monocytes, and granulocytes in the FSC/SSC histograms were used for all list mode files to assure their unattended and automated evaluation. Means from linear or linearized logarithmic analogue-to-digital-converter (ADC) channels as well as absolute cell counts or percent cell frequency values were automatically introduced into data bases (Tables 1, 2).

Triple Matrix Data Base Classification

Data-base data were classified according to the triple matrix concept (Valet et al., 1993) which works in short as follows: Symmetrical upper and lower percentile values (e.g., 15 and 85%) are calculated for the reference value distribution of each database column. All individual data-base column values are then reexpressed as: +, 0, or − according to their position above, within, or below the respective reference percentile thresholds.

A confusion matrix for each classification (disease) state is then set up by the computer program with the different clinical diagnostic or prognostic states on the ordi-

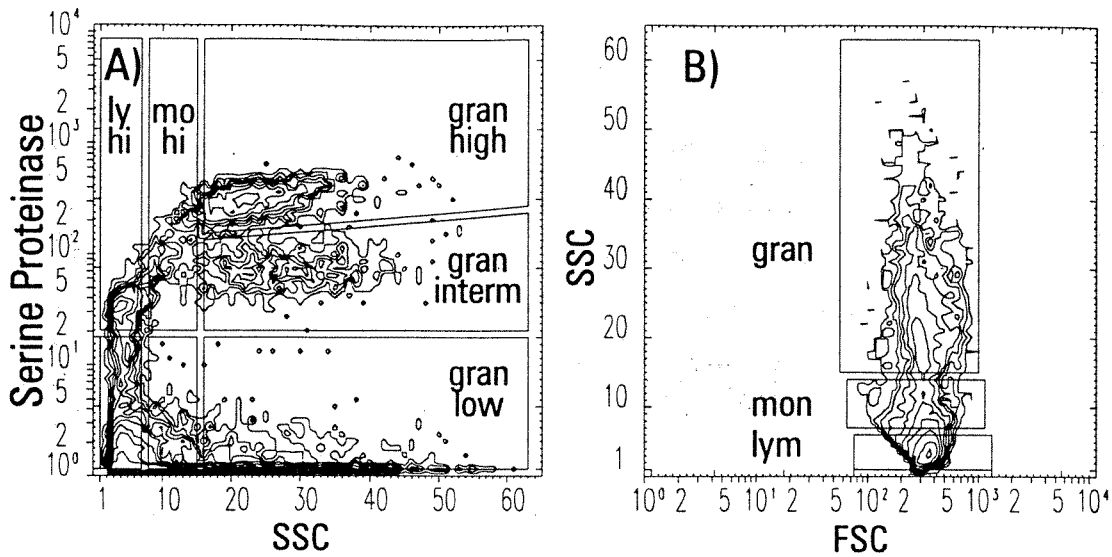

Fig. 2. Standardized evaluation of the $(Z\text{-AlaAla})_2R$ 110 serine proteinase activity measurement of the leukocyte sample of a septic patient. The evaluation of the FSC/R 110 fluorescence and of the FSC/SSC histograms is similar to that in Figure 1. An additional fixed window for the intermediate activity granulocytes is introduced, however, in the FSC/R 110 histogram. Infection and sepsis lead frequently to the formation of a distinct population of vital granulocytes with low serine protease activity (ca 30%). They are CD63 positive; that is, they have secreted a substantial amount of their proteases. The secretion process is paralleled by a significant reduction of SSC.

nate and the corresponding CLASSIF1 classification states on the abscissa. For a correct classification, all diagonal values of the confusion matrix are 100%, with 0% values in the remaining boxes of the confusion matrix.

To achieve as good results as possible, reference classification masks for the various disease states are determined from the learning set in the following way: the most frequent triple matrix character of all samples of a given disease state is introduced for each data-base column into the reference classification mask of this disease. Following the establishment of a reference classification mask for all disease states, all samples of the learning set are individually reclassified according to their highest positional coincidence with the different reference classification masks. Once all samples are classified, the sum of the diagonal values of the confusion matrix is computed to evaluate the quality of the classification. This is followed by the sequential but temporary removal of individual or combinations of data-base columns and by the recalculation of the diagonal sum after each temporary omission step.

Improvement of the diagonal sum means that temporarily eliminated single or combined data-base columns carry no significant classification information, but deterioration of the classification result identifies informative single or combined columns. After all single data-base columns as well as all column pairs and triplets are checked for their information content in all permutations, data-base columns that have not improved the classification result either alone or in conjunction with others are permanently removed, thus leaving the pattern of informative data-base columns in the final reference classification masks of each disease state (e.g., tables 5 and 6 "parameter change" and fig. 5 upper box).

TABLE 1. Columns of DHR 123 Data Base[a]

1 = TOTAL CELLS

Vital Lymphocytes

2 = % LYMPHO
3 = FSC LYMPHO
4 = SSC LYMPHO
5 = SSC/FSC LYMPHO
6 = DHR LYMPHO
7 = DHR/FSC LYMPHO

Vital Low DHR Monocytes

8 = % LOW DHR MONO
9 = FSC LOW DHR MONO
10 = SSC LOW DHR MONO
11 = SSC/FSC LOW DHR MONO
12 = DHR LOW DHR MONO
13 = DHR/FSC LOW DHR MONO

Vital High DHR Monocytes

14 = % HIGH DHR MONO
15 = FSC HIGH DHR MONO
16 = SSC HIGH DHR MONO
17 = SSC/FSC HIGH DHR MONO
18 = DHR HIGH DHR MONO
19 = DHR/FSC HIGH DHR MONO

All Vital Monocytes

20 = % MONO OF VITAL CELLS
21 = FSC ALL DHR MONO
22 = SSC ALL DHR MONO
23 = SSC/FSC ALL DHR MONO
24 = DHR ALL DHR MONO
25 = DHR/FSC ALL DHR MONO

Vital Low DHR Granulocytes

26 = % LOW DHR ACT.VIT.GRANULO
27 = FSC LOW DHR GRANULO
28 = SSC LOW DHR GRANULO
29 = SSC/FSC LOW DHR GRANULO
30 = DHR LOW DHR GRANULO
31 = DHR/FSC LOW DHR GRANULO

Vital High DHR Granulocytes

32 = % HIGH DHR ACT.VITAL GRAN
33 = FSC HIGH DHR GRANULO
34 = SSC HIGH DHR GRANULO
35 = SSC/FSC HIGH DHR GRANULO
36 = DHR HIGH DHR GRANULO
37 = DHR/FSC HIGH DHR GRANULO

All Vital Granulocytes

38 = % GRAN OF ALL VITAL CELLS
39 = FSC ALL DHR GRANULO
40 = SSC ALL DHR GRANULO
41 = SSC/FSC ALL DHR GRANULO
42 = DHR ALL DHR GRANULO
43 = DHR/FSC ALL DHR GRANULO

Dead Cells

44 = TOTAL DEAD CELLS
45 = %TOTAL DEAD CELLS

[a]All values except % and total cell counts represent arithmetic means of linear or linearized logarithmic analogue-to-digital converter (ADC) channel values for all cells in the respective evaluation windows (see Fig. 1B). Columns 12, 18, 24, 30, 36, and 42 represent total cellular oxidative activity whereas columns 13, 19, 25, 31, 37, and 43 are normalized on FSC; that is, they indicate equivalents of intracellular oxidative activity concentration.

Normalization of the learning and test-set data bases as well as of the classification percentiles onto the data-column means of the respective reference samples provides standardized, that is, instrument- and laboratory-independent data bases and classifiers. The measurement of a set of valid reference samples at a new classification site permits the application of a standardized classifier without relearning on abnormal samples, provided that no difference between the normalized new and original reference group has been detected by the CLASSIF1 algorithm.

TABLE 2. Columns of (Z-AlaAla)R 110 Serine Proteinase Data Base[a]

1 = TOTAL CELLS
2 = % VITAL CELLS
3 = % DEAD CELLS
4 = % VITAL LYMPHOCYTES
5 = % VITAL MONOCYTES
6 = % VITAL GRANULOCYTES

Low Serine Proteinase Lymphocytes

7 = % LOW SER LYM/VITAL CELLS
8 = % LOW SER LYM/VIT.LYMPHO
9 = FSC LOW SER LYMPHO
10 = SSC LOW SER LYMPHO
11 = SSC/FSC LOW SER LYMPHO
12 = SER.PROT LOW SER LYMPHO
13 = SER/FSC LOW SER LYMPHO

High Serine Proteinase Lymphocytes

14 = % HIGH SER LYM/VITAL CELLS
15 = % HIGH SER LYM/VIT.LYMPHO
16 = FSC HIGH SER LYMPHO
17 = SSC HIGH SER LYMPHO
18 = SSC/FSC HIGH SER LYMPHO
19 = SER.PROT HIGH SER LYMPHO
20 = SER/FSC HIGH SER LYMPHO

Low Serine Proteinase Monocytes

21 = % LOW SER MON/VITAL CELLS
22 = % LOW SER MON/VIT.MONO
23 = FSC LOW SER MONO
24 = SSC LOW SER MONO
25 = SSC/FSC LOW SER MONO
26 = SER PROT LOW SER MONO
27 = SER/FSC LOW SER MONO

High Serine Proteinase Monocytes

28 = % HIGH SER MON/VITAL CELLS
29 = % HIGH SER MON/VIT.MONO
30 = FSC HIGH SER MONO
31 = SSC HIGH SER MONO
32 = SSC/FSC HIGH SER MONO
33 = SER HIGH SER MONO
34 = SER/FSC HIGH SER MONO

Low Serine Proteinase Granulocytes

35 = % LOW SER GRAN/VITAL CELLS
36 = % LOW SER GRAN/VIT GRANULO
37 = FSC LOW SER GRANULO
38 = SSC LOW SER GRANULO
39 = SSC/FSC LOW SER GRANULO
40 = SER LOW SER GRANULO
41 = SER/FSC LOW SER GRANULO

High Serine Proteinase Granulocytes

42 = % HIGH SER GRAN/VITAL CELLS
43 = % HIGH SER GRAN/VIT GRANULO
44 = FSC HIGH SER GRANULO
45 = SSC HIGH SER GRANULO
46 = SSC/FSC HIGH SER GRANULO
47 = SER HIGH SER GRANULO
48 = SER/FSC HIGH SER GRANULO

[a]All values except % and total cell counts represent arithmetic means of linear or linearized logarithmic analogue-to-digital converter (ADC) channel values for all cells in the respective evaluation windows (see Fig. 2b). Columns 12, 19, 26, 33, 40, and 47 represent total cellular enzyme activity whereas columns 13, 20, 27, 34, 41, and 48 are normalized on FSC; that is, they indicate equivalents of intracellular enzyme activity concentration.

RESULTS

The standardized thresholding during the automated list mode evaluation is quite robust (Figs. 1–3), provided the flow cytometer is daily calibrated to the same sensitivity by monosized fluorescent calibration particles. Although granulocytes and lymphocytes show clear changes in DHR 123 assays following stimulation (Fig. 1A), lymphocytes remain visually close to background. The serine proteinase assay (Fig. 2) displays significant activities for lymphocytes, with usually a high- and low-ac-

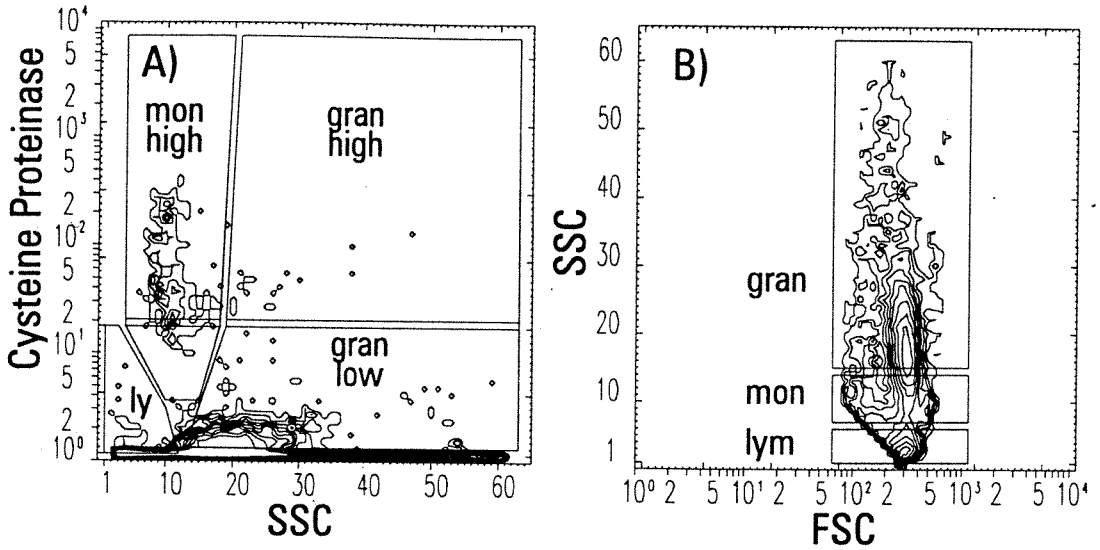

Fig. 3. Standardized evaluation of the (Z-ArgArg)$_2$R 110 cathepsin B,H,L activity measurement of a leukocyte sample from a septic patient. The evaluation is identical to the DHR 123 evaluation of Figure 1.

tivity lymphocyte population (Fig. 2A). Monocytes and granulocytes form a single cluster each in healthy persons. An additional cell cluster of vital intermediate-activity granulocytes at about one-third of the normal activity is seen at variable frequency in infected or septic patients. The low-activity cells are CD63 positive (not shown), indicating that the intermediate-activity granulocytes have secreted intracellular proteases.

Forty-five data-base columns (Table 1) are extracted from each of the four DHR 123 assays, that is, a total of $4 \times 45 = 180$ data-base columns per patient and time point, and 48 data columns (Table 2) + 8 data-base columns for the intermediate-activity cells (not shown) are derived from the serine proteinase as well as 45 data columns for the cathepsin B,H,L (Fig. 3) measurements.

The results from the three assays for the sepsis diagnosis at admission showed that all three assays identify the septic patients in 100% of the cases (Table 3). Because the patient's diagnosis is clinically known, the flow cytometric assay is confirmatory, that is, of little practical interest for the clinician.

The interesting question, however, is whether the assays permit the early identification of nonsurviving patients. Such risk assessment is of great importance for therapy improvement and clearly not available by other clinical or clinical chemistry parameters. If all DHR 123 assay information is used as well as all serine proteinase parameters separately, both assays are capable of predicting the fatal and nonfatal outcome quite correctly on admission, that is, on day 0 (Table 4), although the nonsurviving patients die only between 5 and 14 days later.

This conclusion is reached with a pattern of 11 parameters out of the initial 180 parameters (Table 5). It is unexpected that four DHR parameters are combined with seven scatter, scatter ratio, and percent cell frequency parameters. Furthermore, only three parameters concern granulocytes and three derive from monocytes and five

TABLE 3. Sepsis Diagnosis

Clinical Diagnosis	Patients (*n*)	CLASSIF1 Classification (%)[a]	
		No Infection	Sepsis
	Oxidative Activity		
No infection	15	100.0	0.0
Sepsis	17	0.0	100.0
	Serine Proteinases		
No infection	6	100.0	0.0
Sepsis	23	0.0	100.0
	Cathepsin B, H, L		
No infection	6	100.0	0.0
Sepsis	21	0.0	100.0

[a]25 and 75% percentile thresholds.

from lymphocytes. A similar result is obtained from the 17 parameters of the serine proteinase assay (Table 6) where five serine proteinase activity parameters contrast with 12 scatter and percent cell frequency parameters being distributed into four granulocyte, six monocyte, and seven lymphocyte parameters.

The normalization of all data-base values onto the respective reference values means in each column provides a standardized classifier formula, for example, for the DHR 123 assay (Table 7). The majority of the means of the survivor and nonsurvivor groups are statistically significantly different. The display of the individual values for each of the 11 selected classification parameters for survivors and nonsurvivors does

TABLE 4. Prediction for Sepsis Survival on Admission

Clinical Diagnosis	Patients (*n*)	CLASSIF1 classification (%)	
		Survivors	Nonsurvivors
	A. Oxidative Activity[a]		
Survivor	12	100.0	0.0
Nonsurvivor	5	20.0	80.0
	B. Serine Proteinases[b]		
Survivor	13	100.0	0.0
Non survivor	5	20.0	80.0

[a]25 and 75% percentile thresholds.
[b]20 and 80% percentile thresholds.

TABLE 5. Prediction for Sepsis Survival on Admission: Selected Oxidative Activity Classification Parameters of Table 4A

Parameter	DHR assay	Data column	Parameter change	
			Survivors	Non-survivors
1 = DHR LOW DHR GRANULO	ex vivo	30	0	—
2 = SSC LYMPHO	+ TNF-α	4	0	—
3 = FSC LYMPHO	+ FMLP	3	0	—
4 = % MONO OF VITAL CELLS	+ FMLP	20	0	+
5 = FSC ALL DHR MONO	+ FMLP	21	0	+
6 = DHR/FSC HIGH DHR GRANULO	+ FMLP	37	0	—
7 = DHR/FSC ALL DHR GRANULO	+ FMLP	43	0	—
8 = FSC LYMPHO	+ (T+ F)	3	0	—
9 = SSC LYMPHO	+ (T+ F)	4	0	—
10 = SSC/FSC LYMPHO	+ (T+ F)	5	0	—
11 = DHR/FSC LOW DHR MONO	+ (T+ F)	13	0	+

TABLE 6. Prediction for Sepsis Survival on Admission: Selected Serine Proteinase Parameters of Table 4B

Parameter	Data column	Parameter Change	
		Survivors	Nonsurvivors
1 = FSC LOW SER LYMPHO	9	0	—
2 = SSC LOW SER LYMPHO	10	0	—
3 = SER.PROT LOW SER LYMPHO	12	0	+
4 = SER/FSC LOW SER LYMPHO	13	0	+
5 = FSC HIGH SER LYMPHO	16	0	—
6 = SSC HIGH SER LYMPHO	17	0	—
7 = SER.PROT HIGH SER LYMPHO	19	0	+
8 = FSC LOW SER MONO	23	0	+
9 = SSC LOW SER MONO	24	0	+
10 = SSC/FSC LOW SER MONO	25	0	+
11 = FSC HIGH SER MONO	30	0	+
12 = SSC HIGH SER MONO	31	0	+
13 = SSC/FSC HIGH SER MONO	32	0	+
14 = % LOW SER GRAN/VITAL CELLS	35	0	—
15 = SER/FSC LOW SER GRANULO	41	0	+
16 = % HIGH SER GRAN/VIT GRANULO	43	0	+
17 = SER HIGH SER GRANULO	47	0	—

TABLE 7. DHR 123 Assay: Standardized Means, SEM, and Classification Thresholds of Data Columns Selected for Classification

Data Column[a]	Patients (*n*)	Ex Vivo 30	TNF-α 4	fMLP 3	fMLP 20	fMLP 21	fMLP 37	fMLP 43	TNF-α + FMLP 3	TNF-α + FMLP 4	TNF-α + FMLP 5	TNF-α + FMLP 13
Survivors	13	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
SEM[b]		.108	.044	.035	.144	.039	.059	.212	.031	.035	.020	.035
Non survivors	5	.887	.811	.9961	.9691	.403	.808	.707	.843	.807	.919	1.064
SEM		.196	.051	.151	.540	.336	.257	.389	.059	.057	.025	.124
Classification threshold[c]		<.660	<.928	<.839	>1.600	>1.117	<.731	<.313	<.947	<.929	<.938	>1.224

[a]seeTable.5.
[b]SEM = standard error mean.
[c]Values classified as: − or: + if: < or > as percentile threshold.

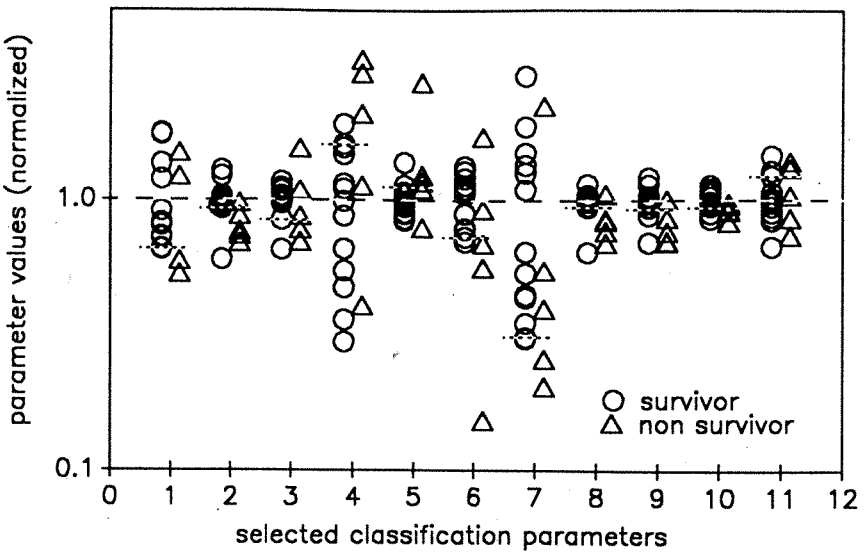

Fig. 4. Display of the selected classification parameters of Table 7 for survivors and nonsurvivors of sepsis. The percentile thresholds are indicated as dotted lines. Values below the threshold are classified as − for percentiles below the 1.0 normalization line while values above the threshold are classified as + for percentiles above the 1.0 normalization line. All other values are classified as 0. Although it seems visually impossible to reach unambiguous classifications according to the aspect of the data clouds, the triple matrix classification of the the same data (Figure 5) is clearly possible. This is due to the inherent error tolerance of the CLASSIF1 classifier for a certain degree of aberrations of the patient sample mask from the reference classification masks.

not look very informative in a traditional display (Fig. 4). The same data as a triple matrix display (Fig. 5), however, show immediately the classification potential of the selected parameters. The classification is error tolerant, as can be seen from the CLAS.COIN data column in which the patient classification mask coincides (1.00) in only 5 of 18 patients with the reference classification masks. Patient 17 is misclassified with nine positional hits for survivor and two hits for nonsurvivor. The plus sign in pattern position 8 counts as a hit for survivor because the nonsurvivor reference classification mask shows a minus sign at this location.

Following the recognition of the usefulness of DHR 123 and serine proteinase assays for individual patient risk assessment in sepsis, it seems important to optimize the number of performed assays. It was checked whether the DHR 123 assay in combination with the serine proteinase assay increased the correct prediction to 100% for survivors and nonsurvivors on the day of admission (Table 4). Furthermore the various DHR 123 assays were checked as single assays as well as in all possible combinations. The results (Table 8) indicate that the combination of the DHR 123 and the serine proteinase assay does not improve the overall result (Table 8, column 4). They also show that the combination of the data from the separate TNF-α and fMLP assays (column 12) classifies even better than all DHR 123 assays together (column 3) with regard to high recognition (100%, upper part) and low multiplicity (1.0, middle part). The optimum percentile threshold in most classifications is 20–80 or 25–75%.

The two unknown test-set patients were correctly classified by the DHR 123 classifier, showing robustness for this restricted number of patients.

NR.	REFERENCE CLASSIFIC.	ABBREVIATION	COIN	REF.CLASSIFIC.MASKS
1	SURV0	aN	1.00	00000000000
2	DEAD0	aT	1.00	---++-----+

REC. NR.	DATAB: S8LEARN.BI4 DATAB.RECORD LABEL	CLASSIF1-CLASSIFIC.	CLAS COIN	SAMPLE CLASSIF.MASKS . = no value
1	KE1277...aN.....	aN	1.00	00000000000
2	KE1292...aN.....	aN	.64	0--0-0+--00
3	KE1296...aN.....	aN	.82	+000+++000+
4	KE1298...aN.....	aN	1.00	+0000+00000
5	KE1299...aN.....	aN	.82	000000-0-00
6	KE1343...aN.....	aN	.64	000+0-000-+
7	KE1344...aN.....	aN	.82	0-0000000-0
8	KE1349...aN.....	aN	1.00	0++-000++00
9	KE1367...aN.....	aN	.91	0000000000+
10	KE1378...aN.....	aN	1.00	00000000000
11	KE1379...aN.....	aN	.73	-00++00000-
12	KE1386...aN.....	aN	.91	0++000-+++0
13	KE1398...aN.....	aN	1.00	0000-0000+0
14	KE1301...aT.....	aT	.55	0--+-++--0+
15	KE1334...aT.....	aT	.73	0-+++----0+
16	KE1376...aT.....	aT	.73	---0+-0----
17	KE1380...aT.....	aN	.82	-000000+0-0
18	KE1382...aT.....	aT	.82	--0++-----0

Fig. 5. Reclassification of the learning set with the selected classification parameters of Table 7. Th learning set classifies quite well with 17 of the 18 patient samples correctly classified (Table 4A). This i quite unexpected if the visual aspect of the same data (Figure 4) is considered. The apparent fact of un ambiguous and mostly correct classification shows the high categorization effect of the triple matrix trans formation of the original data-base parameter values.

DISCUSSION

The results indicate the significant potential of DHR 123 and protease activity deter minations for the risk assessment of surviving and nonsurviving septic patients in in tensive care units. Owing to the restricted number of patients in this pilot study, ad ditional cases are required to establish definitive classifiers for practical use i hospitals. However, according to the present experience, asymptotic classification re sults are usually reached when between 20 and 40 representative patients (Valet et al 1993) are available per classification state.

At this stage, the overall recognition results may decrease but the classifiers es tablished so far have been quite robust upon the addition of new patients. A major rea son for this is probably that all noninformative parameters, that is, the classificatio "noise," are entirely removed during the CLASSIF1 learning process. Additionall the pattern from a certain number of parameters on, for example, >10 parameters, in herently provides a high stability even if some of the parameters have incorrect va

TABLE 8. Optimization of Prediction for Sepsis Survival

Days	Serine Prot.	Oxid All Ass.	All Oxid + Serine	Oxid ex vivo	Oxid TNF-α	Oxid FMLP	Oxid (T+F)	Oxid ex+T	Oxid ex+F	Oxid ex+ (T+F)	Oxid T+F	Oxid F+ (T+F)	Oxid ex+T+F	Oxid T+ F+(T+F)
						A. Average Recognition Index (ARI, %)[a]								
0	90.0	90.0	90.0	90.0	80.0	80.3	90.0	86.2	86.2	90.0	100.0	100.0	100.0	100.0
1	100.0	95.8	95.8	85.8	100.0	100.0	100.0	100.0	100.0	90.0	100.0	95.8	100.0	100.0
2	83.3	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
3	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
						B. Average Multiplicity Index (AMI)[b]								
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.08	1.00	1.00	1.00	1.00
1	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.04	1.00	1.00	1.04	1.04
2	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
3	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
						C. Lower Percentile Threshold (%)[c]								
0	20	25	25	25	20	20	20	25	15	15	25	25	25	25
1	20	25	25	25	20	15	30	30	20	15	30	25	25	30
2	20	25	30	20	20	20	20	25	20	25	25	25	25	25
3	20	25	30	20	20	20	25	25	20	25	25	25	25	20

[a]ARI = sum of recognition values in diagonal of confusion matrix divided by number of classification states (%).

[b]AMI = sum of recognition values in all horizontal lines of the confusion matrix divided by number of classification states and by 100 for data normalization. Optimum 1.00, that is, no multiple classifications (acceptable range 1.00–1.20).

[c]Upper threshold = 100 -- lower percentile (%).

ues. The classifiers are not overdetermined in a statistical sense because the number of classification parameters (Tables 5, 6) is smaller than the total number of classified patients (Table 4). Furthermore, the parameter means (Table 7) of surviving and nonsurviving patients are significantly different in most instances, that is, no "noise" is classified.

The comparison between the traditional data display (Fig. 4, Table 7) and the triple matrix classification matrix (Fig. 5) indicates the importance of regarding flow cytometric results as a pattern rather than as a sequence of single values being compared, for example, by their position in relation to confidence limits. The same was true for the recent evaluation of clinical chemistry values in another intensive care condition, the capillary leak syndrome (CLS) following pediatric cardiac surgery (Tarnok et al., 1997). In this affection, it was impossible to distinguish between the causative role of either surgery or pre-existing humoral or cellular conditions of the organism. CLASSIF1 data pattern analysis clearly indicated a pre-existing humoral or cellular condition, whereas the modalities of surgery seem to be less important. The selected parameter pattern suggests a latent infection as an important CLS risk factor; that is, a preoperatively curable affection is identified by CLASSIF1 data analysis. Pattern analysis in this context provides for the first time individual patient risk assessment, therapy monitoring, and a scientific hypothesis for a difficult clinical condition.

Another important aspect concerns the type of selected classification parameters. Although the usual evaluation in, for example, immunophenotyping, DNA distribution, or thrombocyte activation analysis is preferentially oriented toward the extraction of percent positive/negative, or percent cells in particular evaluation quadrants, the entire CLASSIF1 experience shows so far that the percent cell frequency parameters carry usually only a low information content. This is equally true for the prognosis of septic patients. Only 3 out of 28 parameters (10.7%) listed in Tables 5 and 6 represent percent cell frequency values. The additionally surprising fact in this evaluation is that light scatter parameters contain quite meaningful information although they are in most evaluations utilized only for gating purposes. It is likely that the data pattern evaluation instead of single value analysis will lead to a significant reorientation in the evaluation of flow cytometric data in the future.

The pattern evaluation of the sepsis data provides furthermore efficient means for the optimization of cell assays. Protease and DHR 123 assays provide similar information in this context (Table 4), with the DHR 123 assay being slightly superior (Table 8). The calculation of single and combined assay results in all permutations shows that instead of initially five only two assays, that is, the TNF-α and the fMLP DHR 123 stimulation assay, are really required for the risk assessment of septic patients (Table 8). Both assays indicate the correct prognosis for all patients even on day 0, that is, on admission. The rigorous optimization of cellular assays provides a maximum of information with a minimum of effort.

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