Superiority of a Functional Leukocyte Adhesiveness/Aggregation Test Over the White Blood Cell Count to Discriminate Between Mild and Significant Inflammatory Response in Patients With Acute Bacterial Infections

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Electronic cell counters may underestimate the white blood cell count (WBCC) in the presence of aggregated leukocytes. In the present study we focused on the possibility of using a functional, as opposed to an anatomic, count to circumvent this eventual underestimation. A model of bacterial infection was used because of the importance of leukocytosis in the physician's clinical decision-making process. There were 35 patients with low C-reactive protein (CRP) concentrations (0.5–4.9 mg/dL), 45 with intermediate (5–9.9 mg/dL), and 120 with relatively high (> 10 mg/dL) CRP concentrations. A

significant ($P = 0.008$) difference was noted between the state of leukocyte adhesiveness/aggregation in the peripheral blood of individuals with low CRP concentrations ($3.5\% \pm 4.3\%$) and those with high CRP concentrations ($7.4\% \pm 8\%$), while there was no significant difference in the respective number of WBCs per cubic millimeter (cmm) ($11,600 \pm 5,500$ and $14,000 \pm 7,200$, respectively). We raise the possibility that a functional test might be superior over an anatomic count in patients with acute bacterial infection and a significant acute phase response. *J. Clin. Lab. Anal.* 16:187–193, 2002. © 2002 Wiley-Liss, Inc.

Key words: leukocyte aggregation; bacterial infections; leukocyte adhesiveness

INTRODUCTION

The white blood cell count (WBCC) is the most used laboratory test for patients with acute bacterial infections. Most clinicians assume that a significant leukocytotic response is associated with a more significant inflammatory response, and vice versa. However, the development of tissue leukostasis or the presence of pseudoleukopenia may be associated with an underestimated leukocyte count (1–3).

In the present study we used a quantitative C-reactive protein (CRP) measurement as a standard laboratory marker to assess the intensity of the inflammatory response. The results reported herein indicate that a

functional, as opposed to an anatomical, particle count might be superior for the detection of enhanced inflammatory response in individuals with an acute bacterial infection. Possible explanations for this superiority are discussed.

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MATERIAL AND METHODS

Subjects

All patients and controls gave their consent for participation according to the guidelines of the local ethics committee. We included patients with acute bacterial infection who had been admitted to the Department of Internal Medicine "D" at the Tel-Aviv Sourasky Medical Center. Blood was obtained during the first 24 hr of their stay in the hospital. The following conditions were considered as having a bacterial etiology: lobar pneumonia, sepsis with positive blood cell cultures, cellulitis, erysipelas, abscess, infected pressure sores, streptococcal tonsillitis, urinary tract infections, and/or pyelonephritis with positive urinary cultures, meningitis, and pseudomembranous colitis. Excluded from the present study were 23 individuals with an underlying inflammatory or malignant disease, and patients who were on steroidal or nonsteroidal anti-inflammatory medications except for low-dose (<325 mg/day) aspirin. The remaining patients with bacterial infections were arbitrarily divided into three groups according to the CRP concentration: "low" was defined as a concentration of 0.5–4.9 mg/dL, "intermediate" as 5–9.9 mg/dL, and "high" as >10 mg/dL. Healthy members of the medical staff, as well as volunteers from a health club, agreed to take part in the study and served as controls.

Laboratory Methods

Variables of the acute phase response

The WBCC and differential were determined by using the STKS Coulter electronic cell analyzer (Elkhart, IN, USA). The erythrocyte sedimentation rate was performed by the method of Westergren, the concentration of fibrinogen by the method of Clauss, and the quantitative CRP by nephelometry and specific anti-human CRP antibodies. The cut-off point for positivity for CRP in our laboratory is 0.5 mg/dL.

Leukocyte adhesiveness/aggregation test (LAAT)

In vivo leukocyte aggregation in the peripheral blood was assessed by the leukocyte adhesiveness/aggregation test (LAAT) (4). One drop of citrated blood (one volume of 3.8% sodium citrate and three volumes of whole blood) was placed on a slide that was held for 2–3 min at a 45° angle so that the blood would be pulled down by gravity, leaving a fine film. The slide was then dried at room temperature in a horizontal position. Automatic staining was performed with the HEMA TEK slide stainer (AMES, Cedax, France) and a HEMA TEK bloc colorant stain pack (Bayer Diagnostics, Cedax, France).

Inflammation meter (IM)

The image analysis system (INFLAMETTM, Inflomet Ltd; Tel Aviv, Israel) (5–10) consists of a Pentium computer running Windows 95, equipped with a Matrox Meteor color frame grabber, a color CCD camera, and a microscope that is operated at a $\times 200$ magnification, resulting in an image resolution of 0.4 μ per pixel. Nine images are taken from each slide. The fields of view are chosen systematically to sample different regions on the slide. Each image is processed separately and the outputs are then averaged to form the final slide outputs. The nine fields of view cover a total area of 0.6 mm.

The IM application software detects WBCs and sorts them into clusters. Detection of cells is based on their color, shape, and size. It was of special importance for us to correctly detect and classify WBCs even when they were in close proximity. We therefore took special care to develop an algorithm that avoids errors which result in missing or merging of some leukocytes, or rejection of a whole cluster as a speck of dirt.

For cluster analysis, two leukocytes are considered as being near to each other if the distance between their centers is less than 10 μ s. The aggregation level is defined as the percentage of leukocytes in clusters of size >1. Data related to variabilities of this technique are given elsewhere (8).

Whole blood flow cytometry

All blood samples from patients and controls were obtained between 08:00 and 11:00 AM. Time to processing was similar in all patients and varied between 30–90 min. All preparative steps were done at room temperature so as to minimize potential changes in the expression of the leukocyte's integrin (11). Cellular surface expression of the adhesion molecules CD11b/CD18 and CD62L (L-selectin) was measured by a direct immunotechnique of flow cytometry. Saturation concentrations of the FITC-labeled monoclonal antibodies to either the CD11b/CD18 or the CD62L antigen were added to 50 μ L of venous blood that was drawn into EDTA-containing polystyrene round-bottom tubes (FALCON 2050-Becton Dickinson Labware Franklin Lakes, IN, USA) and incubated at room temperature for 10 min in the dark. Fixation and hemolysis were then performed in a Coulter 2 Prep (Epics immunology work station) by using the Coulter 2 Prep Epics system (Coulter Corporation, Hialeah, FL). This technique avoided in vitro manipulation, which could have activated the cells. We did not wash the cells following processing in the Coulter Q-Prep system to avoid the potential loss of activated cells during this procedure. Samples were then run through a FACscan flow

cytometer (Becton-Dickinson) to measure the fluorescent signal. Gating of the forward and side scatter diagrams was used to pinpoint the analysis to a homogeneous cell population, excluding clusters, debris, and red cell fragments. The CD45 FITC and CD14 PE monoclonal antibodies (Dade Behring, Frankfurt, Germany) were used to verify that we were indeed looking at our target cell population and excluding fragments of red blood cells. The laser excitation wavelength was 488 nm and the emission light was 530 nm. A frequency distribution histogram of the fluorescent signal was obtained from at least 5,000 cells for each sample with a Hewlett-Packard computer programmed with LYSYS II profile analyzer software. Negative control for non-specific flow cytometric background was performed with an isotype-matched, fluorescent monoclonal antibody. Neutrophils could be distinguished from lymphocytes and monocytes by the combination of low-angle forward and right-angle scattered laser light, and a cytogram of each cell population was generated. An observer using a computer-aided outlining technique identified the region of interest in each population of neutrophils, monocytes, or lymphocytes. A histogram of fluorescence distribution was constructed with fluorescence intensity displayed on the abscissa and the number of cells on the ordinate. The relative mean channel fluorescence (MCF) was obtained from the histogram and expressed as an index of membrane surface expression. Incubating control antibodies and determining the nonspecific fluorescence set the cut-off point for positivity.

The parameters of the flow cytometer did not change significantly during the study, as verified by means of calibration beads (quantum fluorescence kits; Flow Cytometric Standard Corp., San Juan, PR). There was no significant change in the reading of the standard beads from one day to the next; the mean $\pm$ SD MCF for the four beads was 78 ± 9 , 108 ± 7 , 125 ± 6 , and 147 ± 5 ($n = 93$), respectively. This methodology permits the comparative analysis of patients examined during different days.

Monoclonal antibodies. All of the monoclonal antibodies were purchased from Immunotech (Marseille, France). The CD62L FITC was an IgG1 monoclonal antibody (dreg 56) that recognizes the L-selectin antigen on the leukocytes. The CD11b FITC was an IgG1 antibody that recognizes the heterodimer glycoprotein of 165 kDa (α chain) and 95 kDa (β chain) (the MAC-1 antigen). Negative control for nonspecific flow cytometric background was performed with an isotype-matched, fluorescent, nonbinding monoclonal antibody.

Statistical Analysis

The statistical analysis was performed with the SPSS (SPSS Inc., Chicago, IL, USA) package.

RESULTS AND DISCUSSION

We examined a total of 200 patients (109 men and 91 women; mean $\pm$ SD age = 66 ± 22 years) with various bacterial infections. In addition, there were 49 healthy controls (25 men and 24 women; mean $\pm$ SD age = 52 ± 21 years). The patients' diagnoses are listed in Table 1. The laboratory results of the patients, who had been categorized into three groups of inflammatory response intensity according to CRP concentrations, and those of the controls are reported in Table 2. While all variables of the acute phase response, including the LAAT, could differentiate between the presence or absence of an acute phase response, the total WBCC could not distinguish between patients with a low CRP concentration and those with a relatively high CRP concentration. We also measured the strength of correlation between the different variables and the CRP group using the Spearman correlation coefficient (Table 2). We found that the coefficient was higher for the LAAT than for WBCC. Moreover, we performed a discrimination analysis to distinguish between the mild-infection group and the severe-infection group. Using the WBCC, 54% of the cases were correctly classified, whereas 64% were correctly classified using the LAAT. The Pearson correlation coefficient between the LAAT and the WBCC was significant ($R = 0.56$, $P < 0.0001$). A graphic presentation of the mean $\pm$ SD of the WBCC and LAAT vs. the four subgroups of no, mild,

TABLE 1. Number of patients in each diagnostic category according to C-reactive protein (CRP) concentration

Diagnosis	CRP concentration		
	Low ($n = 35$)	Intermediate ($n = 45$)	High ($n = 120$)
Pneumonia	6	8	36
Urinary tract infection and pyelonephritis	13	18	34
Cellulitis, abscess and erysipelas	7	10	20
Sepsis	1	3	13
Infected pressure sore	2	3	9
Pseudomembranous colitis	2	2	5
Tonsillitis	3	1	—
Cholecystitis	—	—	3
Meningitis	—	—	3
Osteomyelitis	—	—	3
Prostatitis	—	—	1
Bacillary dysentery	—	—	3
Active tuberculosis	1	—	—

TABLE 2. Values (mean $\pm$ SD) of the various acute phase response reactants in the controls and in patients with bacterial infections*

	WBCC (cells/cmm $\times 10^3$)	PMN%	MONO%	LY%	ESR (mm/H)	Fg mg/dl	LAAT(%)	Absolute PMN
Control (n = 49)	7 $\pm$ 2.2 <i>P</i> < 0.00001	59 $\pm$ 9 <i>P</i> < 0.00001	8 $\pm$ 5 NS	29 $\pm$ 9 <i>P</i> < 0.00001	14 $\pm$ 13 <i>P</i> < 0.00001	302 $\pm$ 86 <i>P</i> < 0.00001	0.4 $\pm$ 0.7 <i>P</i> < 0.00001	4.4 $\pm$ 2 <i>P</i> < 0.00001
Low CRP (n = 35)	11.6 $\pm$ 5.5 NS	78 $\pm$ 11 NS	7 $\pm$ 4 NS	12 $\pm$ 8 NS	35 $\pm$ 25 <i>P</i> = 0.0005	455 $\pm$ 141 <i>P</i> = 0.02	3.5 $\pm$ 4.3 <i>P</i> = 0.03	8.6 $\pm$ 4.6 NS
Intermediate CRP (n = 45)	12.3 $\pm$ 5.8 NS	80 $\pm$ 12 <i>P</i> = 0.0006	7 $\pm$ 3 <i>P</i> = 0.004	11 $\pm$ 10 <i>P</i> = 0.007	57 $\pm$ 29 NS	534 $\pm$ 135 <i>P</i> = 0.003	6.2 $\pm$ 6 NS	9.4 $\pm$ 4.9 NS
High CRP (n = 120)	14 $\pm$ 7.2 NS	85 $\pm$ 7 <i>P</i> < 0.00001	5.3 $\pm$ 3 <i>P</i> = 0.006	8 $\pm$ 5 <i>P</i> = 0.0005	64 $\pm$ 30 <i>P</i> < 0.00001	615 $\pm$ 155 <i>P</i> < 0.00001	7.4 $\pm$ 8 <i>P</i> = 0.008	12 $\pm$ 6.7 <i>P</i> < 0.0001
High vs. Low CRP	NS	<i>P</i> < 0.00001	<i>P</i> = 0.006	<i>P</i> = 0.0005	<i>P</i> < 0.00001	<i>P</i> < 0.00001	<i>P</i> = 0.008	<i>P</i> < 0.0001
Spearman Correlation	<i>r</i> = 0.50	<i>r</i> = 0.64	<i>r</i> = -0.33	<i>r</i> = -0.61	<i>r</i> = 0.57	<i>r</i> = 0.62	<i>r</i> = 0.56	<i>r</i> = 0.57
Coefficient	<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> < 0.0001	<i>P</i> < 0.0001

*The Spearman Correlation Coefficients between the variables and the CRP group are presented.

The values were divided arbitrarily into mild, intermediate or intense inflammatory response groups according to the concentration of the C-reactive protein. Reported as well are the results of the T test between the controls and the groups of low CRP, between low and intermediate CRP, between intermediate and high CRP as well as between low and high CRP concentration. WBCC, white blood cell count; PMN, polymorphonuclears; MONO, monocytes; LY, lymphocytes; ESR, erythrocyte sedimentation rate; Fg, fibrinogen; LAAT, leukocyte adhesiveness/aggregation test; CRP, C-reactive protein; NS, not significant.

wbcc and laat in the four subgroups

Fig. 1. A graphic presentation sharing the mean $\pm$ SD of the WBCC and LAAT vs. four subgroups of no (1), mild (2), moderate (3), and severe (4) infection/inflammation.

moderate, and severe infection/inflammation is depicted in Fig. 1. Typical examples of pictures obtained from a control, a patient with urinary tract infection and a low CRP, a patient with erysipelas and an intermediate CRP, and a patient with staphylococcal sepsis and a high CRP are displayed in Fig. 2.

In the present study, we determined the availability of two adhesive proteins, namely the CD11b/CD18 and CD62L antigens. We wanted to find out whether the increased leukocyte adhesiveness in our patients with acute bacterial infection resulted from an increased expression of these proteins on the surface of the cells.

Fig. 2. Typical pictures obtained by the Inflammation Meter (IM) from a healthy volunteer (upper left), a patient with a urinary tract infection and a CRP concentration of 3.2 mg/L (upper right), a patient

with erysipelas and a CRP concentration of 7.6 mg/L (lower left), and a patient with staphylococcal sepsis and a CRP concentration of 19.6 mg/L (lower right).

TABLE 3. The availability of the CD11b/CD18 and CD62L antigens on the surface of the peripheral blood leukocytes (in units of mean fluorescence intensity, MFI) in patients with bacterial infections and in the controls*

	Polymorphonuclears		Monocytes	
	CD11b/CD18	CD62L	CD11b/CD18	CD62L
Controls (n = 49)	161 ± 117	235 ± 61	198 ± 77	195 ± 57
Low CRP (n = 35)	209 ± 137	207 ± 81	321 ± 150	272 ± 147
Intermediate CRP (n = 45)	205 ± 92	208 ± 61	293 ± 91	272 ± 103
High CRP (n = 120)	205 ± 125	231 ± 102	296 ± 110	301 ± 110

*Patients with bacterial infections were categorized according to intensity of the inflammatory response according to CRP concentration. CRP, C-reactive protein.

The results (Table 3) show that there was a significant ($P < 0.00001$) increment in the expression of these molecules on the surface of the mononuclear cells in

the group of patients as a whole, with no significant differences between the subgroups of CRP concentrations. While there was a slight increment in the availability of the CD11b/CD18 antigen on the surface of the peripheral blood polymorphonuclear leukocytes of the patients, this difference did not reach statistical significance.

The differentiation between patients with acute bacterial infection and significant inflammatory response and those with a less prominent infections is a daily clinical problem. Most physicians use clinical signs and symptoms, but laboratory tests might be helpful as well. The WBCC is probably used most by clinicians for this purpose. However, the presence of increased cell activation and aggregation during bacterial infections, and especially in the presence of more severe infections, may be associated with both tissue leukostasis and electronic counter-related pseudoleukopenia (1–3). This pseudoleukopenia is a result

of the presence of aggregated WBCs in the peripheral blood (3).

It is our opinion that the disadvantage of leukocyte aggregation-related pseudoleukopenia can turn into a diagnostic advantage, once the percentage of peripheral blood aggregated leukocytes is taken into consideration. In fact, it has been shown repeatedly, by various research groups, that the LAAT is a sensitive marker for the detection of the acute phase response, as well as for the assessment of its intensity (12–13). This test retains its diagnostic potential in patients with bacterial infections (14–18) and has been shown to correlate significantly with sensitive markers of the acute phase response, including CRP, IL1, IL6, and IL8 in a model of bacterial meningitis (19).

In the present study we used quantitative CRP concentrations as a standard laboratory test that was related to the intensity of the inflammatory response. The division into the three groups of CRP intensity was arbitrary. Nevertheless, most clinicians will agree that a CRP concentration below 5 mg/dL represents a relatively low acute phase response, while those above 10 mg/dL are representative of a significant response. Our results show that while it is possible to differentiate between the two groups of relatively low and relatively enhanced inflammatory response by using the LAAT, this could not be done with similar effectiveness by using the total WBCC.

In our opinion, this indicates that the LAAT is a more sensitive test. An alternative explanation involves the presence of relatively low leukocyte counts in individuals with significant bacterial infections. The reason for such a relatively reduced count could be related either to the phenomenon of tissue leukostasis or to an electronic counter-related pseudoleukopenia in the presence of aggregated leukocytes.

In this work we used the availability of both CD11b/CD18 and CD62L antigens on the surface of peripheral blood leukocytes as markers of cell activation. It was found that they were enhanced on the cells of the patients as compared to those of the controls. However, discrimination between the two groups of relatively low and relatively enhanced inflammatory response was not possible—probably because the more activated cells stuck to the endothelium and were not present in the circulation pool of peripheral blood.

Finally, we turn to the question of availability of the LAAT. We have recently developed special image analysis techniques that permit the identification of leukocytes as well as the determination of the degree of their adhesiveness/aggregation within a couple of minutes, and at extremely low cost (21). These techniques may help make the LAAT a clinically useful diagnostic tool.

CONCLUSIONS

We conclude that it is possible to use the LAAT as a marker of significant inflammatory response in individuals with an acute bacterial infection. Recent reports (17–19,20) of the usefulness of this test in differentiating between viral and bacterial infection may add to its clinical attractiveness.

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