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VIABILITY OF AUTOLOGOUS HUMAN LEUKOCYTES LABELED BY ^{99m}Tc -HMPAO

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VIABILITY OF AUTOLOGOUS HUMAN LEUKOCYTES LABELED BY ^{99m}Tc -HMPAO

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Abstract

Radiolabeling and re-injection of autologous leukocytes is a nuclear medicine technique used in the scintigraphic detection of hidden infections. Since the labeled leukocytes have to be re-injected into the patient, strictly aseptic conditions are required for the labeling procedure. In the present paper the separation and labeling of mixed leukocytes with ^{99m}Tc -HMPAO were performed by using a closed, disposable kit, that, simplifying the preparation process, minimizes the possibility of accidental microbiological and radioactive contamination of the sample and operator. The recommended European procedure recommends that, if the labeling yield is <40%, further quality controls should be mandatorily performed, such as microscopic inspection and trypan blue exclusion test for cell viability. In a cohort of 280 patients the determination of leukocyte viability was performed for every examined patient, in order to ensure the best clinical significance of the scintigraphy and assure a stringent respect of Good Medical Practice. As the absolute values of leukocyte viability before the labeling demonstrated high patient variability, an accurate examination of collected clinical data was performed, in the attempt to explain this high dispersion of values. The non viable/viable leukocyte ratio BEFORE radioactive labeling was taken into consideration in the present work, and a possible relation with other patient parameters or clinical factors was studied. A direct correlation between leukocyte viability and infection severity was found, and a clinical explanation is briefly discussed.

Keywords: *Leukocytes - White blood cells - ^{99m}Tc -HMPAO – Radioactive labelling*

Riassunto

La marcatura radioattiva e la re-iniezione di leucociti autologhi è una tecnica di medicina nucleare utilizzata nella rilevazione scintigrafica di infezione nascoste.

Poiché i leucociti marcati devono essere re-iniettati nel paziente, è necessario operare in condizioni strettamente asettiche per la procedura di marcatura. Nel presente lavoro la separazione e la marcatura con ^{99m}Tc -HMPAO sono state eseguite utilizzando un sistema chiuso, in kit monouso che, semplificando il processo di preparazione, riduce al minimo la possibilità di una contaminazione microbiologica o radioattiva accidentale del campione e dell'operatore.

Le procedure europee prevedono che, nel caso di resa di marcatura <40%, ulteriori controlli di qualità debbano essere effettuati, come ad esempio l'ispezione al microscopio e il test di esclusione del tripan blu per la vitalità cellulare. In un gruppo di 280 pazienti è stata eseguita la determinazione della vitalità leucocitaria in ogni paziente esaminato, al fine di garantire la miglior significatività clinica della scintigrafia ed assicurare un rigoroso rispetto della Good Medical Practice.

Poiché i valori assoluti di vitalità dei leucociti della marcatura hanno evidenziato una elevata variabilità tra paziente e paziente, è stato eseguito un più accurato esame dei dati clinici raccolti, nel tentativo di spiegare questa elevata dispersione dei valori. Il rapporto leucociti non vitali/leucociti vitali PRIMA della marcatura radioattiva è stata presa in considerazione nel presente lavoro, ed è stata studiata una sua possibile relazione con altri parametri del paziente. Una correlazione diretta tra vitalità dei leucociti e gravità dell'infezione è stata riscontrata, e una interpretazione clinica è stata brevemente discussa.

Parole chiave: *Leucociti – Globuli bianchi - ^{99m}Tc -HMPAO – Marcatura radioattiva*

INDEX

Introduction	5
Leukocyte collection and labeling	6
Leukocyte viability test	6
Results	7
Discussion	14
References	20

Introduction

Radioactive labeling and reinjection of autologous leukocytes is a technique currently used for the scintigraphic detection of soft tissue or musculoskeletal infection, due to the characteristic of leukocytes to accumulate in inflammatory foci.

As ^{99m}Tc is currently the preferred radioisotope in nuclear medicine, due to its ideal nuclear and chemical characteristics and its low cost, many ^{99m}Tc labeled molecules have been tested and used for leukocyte labeling (1-3).

The molecule ^{99m}Tc -HMPAO (hexamethylpropyleneamine oxime - $^{99m}\text{Tc(III)}$) is a complex developed for brain scintigraphy; due to its lipophilic characteristics, the molecule has the ability to cross the blood-brain barrier. This complex has also the ability to penetrate through the leukocyte membrane by passive diffusion, is retained and converted into the cells in a hydrophilic complex (4), and is currently the most widely used molecule for white blood cells labeling in clinical practice. In normal individuals about 60% of the radioactivity associated to labeled leucocytes is taken by the liver, spleen, bone marrow, followed by an exponential clearance from the blood (5).

Clearance of labeled leucocytes from liver and spleen is rather slow, while the pulmonary activity is rapidly eliminated; an increasing lung leukocyte uptake extending for 30 min, or more, is generally considered a clear indication of cell damage or activation of leukocytes, and the clinical examination results can be questionable. By using controlled ^{99m}Tc -HMPAO labeling procedure, leukocyte recovery in different experiments reached $82\% + 6$ (n=820) (6).

The majority of patients undergoing WBC scintigraphy (particularly in our Nuclear Medicine Division) are examined for suspected septic infections of joint replacement. In these cases a standard acquisition protocol includes planar segmental acquisition approximately within 20-30 minutes after injection, a second planar segmental acquisition 2-4 hours p.i., associated with a total body scan and, finally, an

acquisition in the second day of the planar static scintigraphy, associated with a second total body scanning.

Leukocyte collection and labeling

White cell concentrates were obtained starting from the blood of patients, according to the following procedure: a 60 ml disposable syringe containing 8 ml of ACD-A (Acid Citrate-Dextrose Anticoagulant) solution was filled with 32 ml of whole blood. To this syringe 10 ml of 10% 2-hydroxyethyl starch (HES) pharmaceutical grade, molecular weight 200 kDa, were added, the content was gently mixed, and the syringe was kept in vertical upside position to allow the erythrocytes sedimentation. After 2 hours, the leukocyte rich supernatant fraction was collected, transferred in a second syringe, and centrifuged at 150g for 5 min. After removal of the plasma, the leukocyte pellet was re-suspended and incubated with a solution of freshly prepared ^{99m}Tc -HMPAO in saline, with an HMPAO concentration of 125 $\mu\text{g/ml}$.

After 15-20 min, 5 ml of saline were added, the suspension was centrifuged at 150g for 5 min, and the pellet, re-suspended in saline, was re-injected to the patient. The sedimented erythrocytes and leukocyte depleted plasma fractions were discarded. All the separation and labeling steps were performed by means of a disposable, ready-to-use closed system kit, containing all necessary sterile materials and reagents (WBC Marker Kit, Celltech, Turin, Italy).

Leukocyte viability test

Equal volumes (10 microliters) of labeled leukocytes cell suspension, ready for patient injection, and of diluted trypan blue solution in saline (0.4%) were put into

contact and mixed, and the resulting solution was visualized under a digital microscope (500X) for counting of blue-stained cells (cell viability test). The total leukocyte were visualized by mixing 10 microliters of labelled leucocytes suspension with 150 microliters of acridine orange solution, and visualized under the same microscope. An image analysis software, based on the open source Image-J NIH package, was used for the automatic counting of total and non-viable leukocytes, and of erythrocytes in the microscope optical field.

All the colorimetric microscopy viability test was performed by means of a disposable, ready-to-use system kit, containing the dedicated microscope (500x), and all necessary materials and reagents (Leukocyte Viability Kit, Celltech, Turin, Italy). A second confirmation viability test was randomly performed, by putting in contact equal volumes of labeled leucocytes suspension and of a mixture acridine orange/ethidium bromide in saline, both 10 µg/ml, and counting the ratio non viable/viable cells under a Zeiss fluorescence microscopy .

Results

The European approved procedures (7) recommends that if the labeling yield is <40%, further quality controls should be performed, such as microscopic inspection and trypan blue exclusion test for cell viability. Usually a 0.4% trypan blue solution is mixed with the labeled leucocyte cell suspension, and examined under a phase-contrast microscope at 100-fold magnification, by counting the percentage of blue-stained cells (cells that have been damaged during the labeling process). As a control, the same procedure is repeated by using unlabeled leucocytes. A preparation with >4% of dead cells (blue-stained cells) after labeling should not be released for injection into the patient.

The procedure used in the present work differ slightly from the one generally used in the viability test, as concentrated acridine orange is used for the total leukocytes

determination. Moreover, the characteristics of the microscope also allows the routinary quantitative determination of red blood cell in the trypan blue stained leukocytes suspension.

In Figure 1 is reported a typical microscope image obtained after a staining of the sample of radiolabeled leukocytes with acridine, immediately after the labeling; each yellow point marks a single leukocyte.

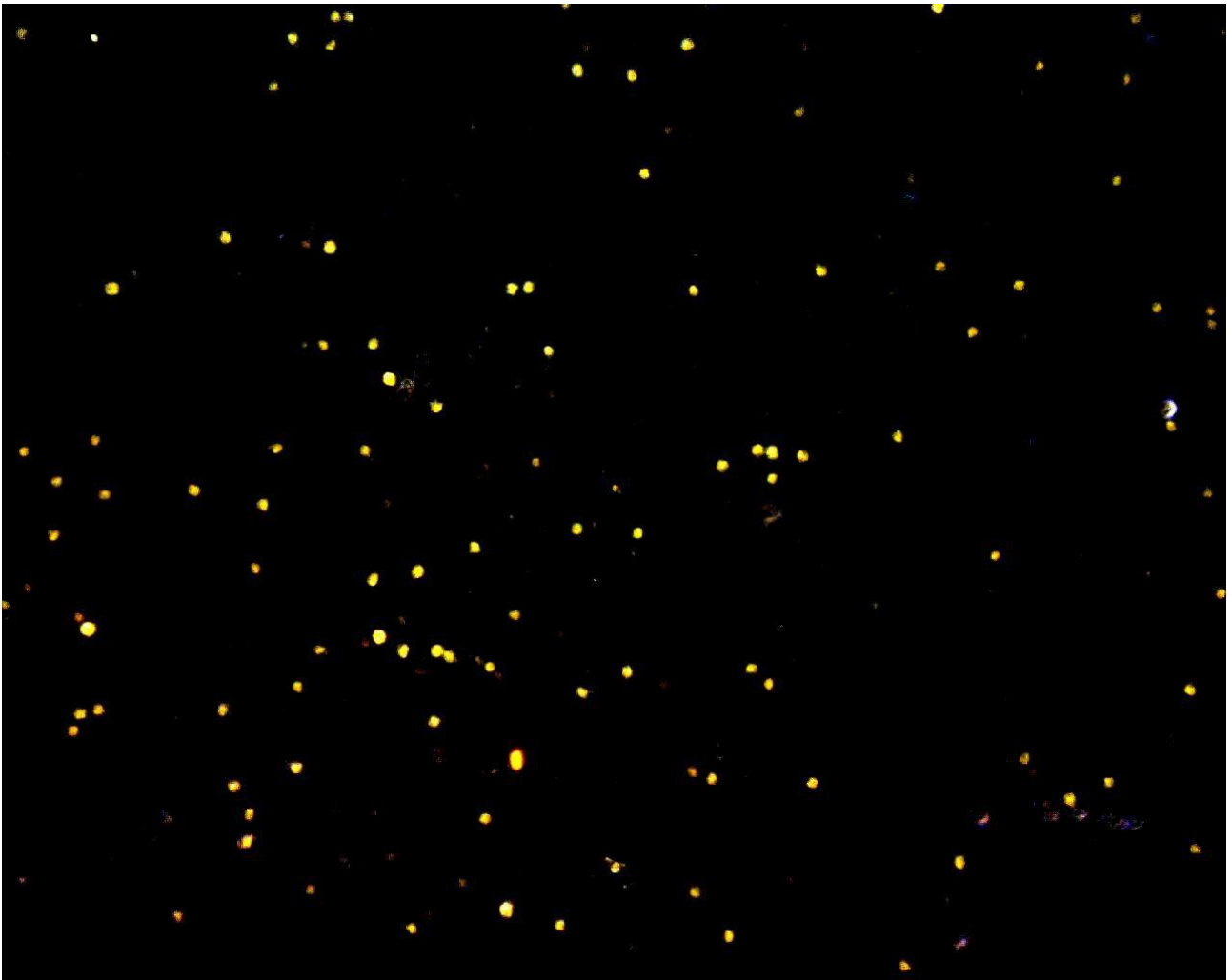


Figure 1

The Figure 2 shows the microscopy field after trypan blue staining on the same sample, immediately after labeling; non viable leukocytes are indicated by the blue spots, while the RBC are clearly visible as red points.

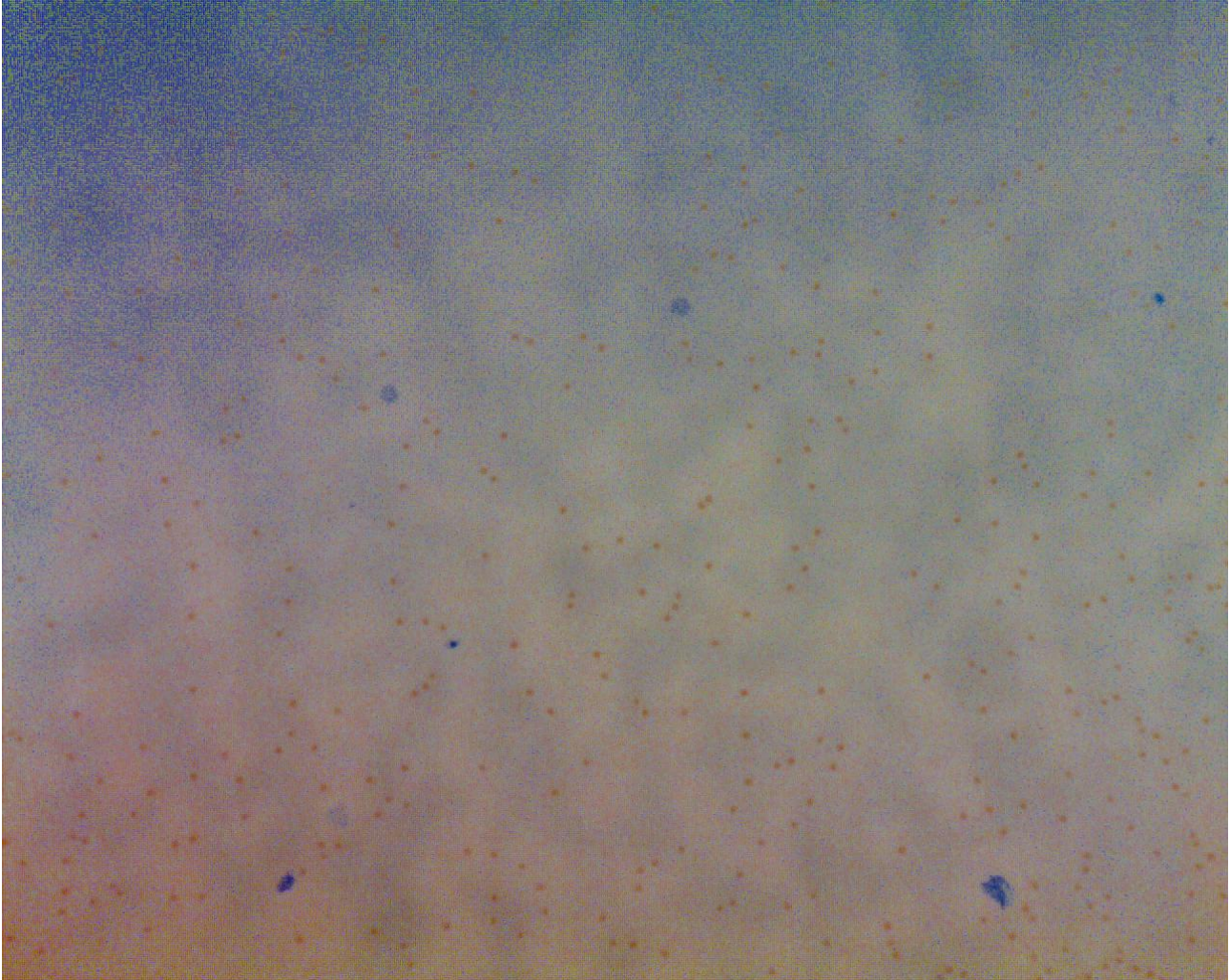


Figure 2

In the operative procedure of our Nuclear Medicine Division the determination of leukocyte viability is performed for every examined patient, in order to ensure the clinical significance of each scintigraphy and assure a stringent respect of Good Medical Practice. The injection of labeled leukocytes suspension was always performed within 30 minutes from end of labeling, and a mean value of non-viable leukocyte after labeling, of $1.1\% \pm 0.3$ was obtained ($n = 280$). This amount

represents the difference between non viable leukocytes before and after radioactive labeling, and can be basically considered an indication of the quality of the preparation. The absolute values of leukocyte viability before the labeling were however rather different from patient to patient, and showed an high individual variability. In the attempt to explain this high dispersion of values, the viable/non viable leukocyte ratio BEFORE radioactive labeling was taken into consideration in the present work, and a possible correlation with other patient parameters or factors was attempted, by choosing a cohort of patients, in which a complete clinical history was present.

As a first, a graphic of % Non-viable leukocyte (R) *vs* Age of the patients was plotted, and is shown in Figure 3.

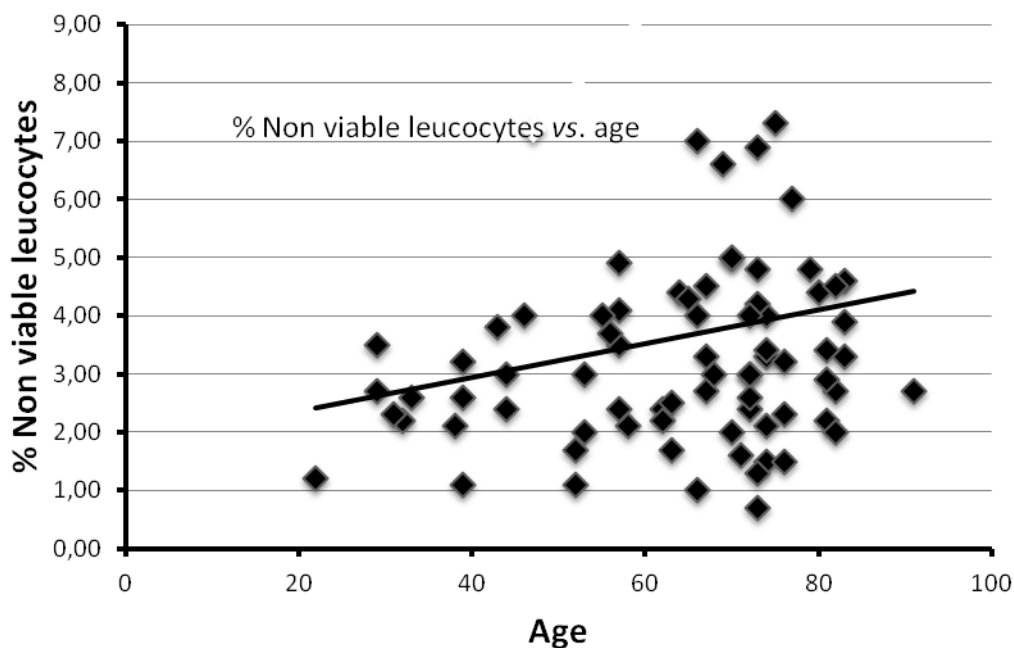


Figure 3

Although the cluster presents an high variability, it is clearly visible an increase of the % of non-viable leucocytes with the increase of the age of the patients. The result is

not surprising, as aging is associated with impairment (in number and function) of a wide diversity of cell types, including those from the immune system.

Telomere lengths in peripheral blood leukocytes from 75 patients of various age have been measured. From birth through about age 4 a rapid decline in average telomere length was observed, and then telomere lengths appear to remain stable until early adulthood. The final phase involves a gradual decline in mean telomere length that is associated with advancing age (8).

In order to examine the possibility that the infection status is correlated to the leukocyte viability, the same cohort of patients was divided in four groups:

(1) patients in which the labeled leukocyte scintigraphy didn't showed any specific body uptake

(2) patients in which the selective body uptake in early images, generally of low intensity, was attributed to an inflammation

(3) patients in which the labeled leukocyte scintigraphy demonstrated a selective, specific body uptake, attributed to infection. The majority of these patients have been found positive for septic infections of joint replacement, and a minority for vascular prosthesis septic infections.

(4) patients in which the labeled leukocyte scintigraphy demonstrated a selective, specific body uptake, attributed to infection, AND that at the moment of the scintigraphic were under antibiotic treatment from less than two weeks.

Results are summarized in Figure 4, and clearly demonstrate that, while a common inflammation is not able to modify the leukocyte viability status, an infection has a marked effect in decreasing the leukocyte viability; the antibiotics, used in presence of an infection, only slightly increases this viability. No observable difference was seen between orthopedic and vascular septic infection.

The viability seems rather correlated with severity of infection; inflammation, which in most these patients can be attributed to mechanical reason (arthrosis, irritation from prosthesis, vascular involvement) doesn't involve a leukocyte active role, and consequently no visible long term leukocyte recruitment is revealed in the scintigraphic image; this fact also accounts for the lack of variation in leukocyte viability.

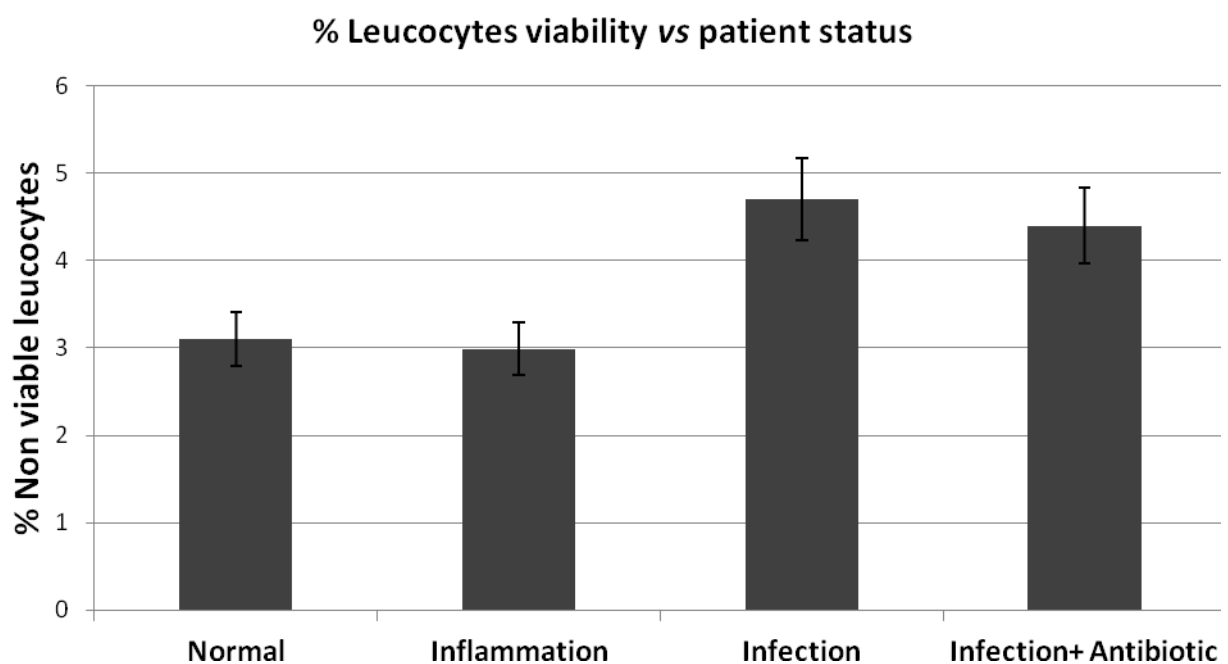


Figure 4

In literature it has been demonstrated (9) an in-vitro effect of killed *Staphylococcus aureus* cells on bovine blood mono-nuclear leukocytes from uninfected cows, assessed using a lymphocyte proliferation assay and a ^{51}Cr release cytotoxicity assay. Killed *S. aureus* cells cultured with mononuclear leukocytes caused a concentration-dependent decrease in lymphocyte proliferation that was associated with a concomitant decrease in mononuclear leukocyte viability.

It is known that *Escherichia coli* alpha-hemolysin has a role in pathogenesis, and its effect on human peripheral leukocyte viability was studied in vitro (10). Viability of leukocytes exposed to low doses of alpha-hemolysin decreased nearly 10 fold within 15 min of exposure. This response was dose and time dependent and was neutralized by antiserum, heat, proteases, and lipase. To gain further evidence that alpha-hemolysin was the molecule responsible for leukocytes toxicity, preparations of alpha-hemolysin were passed through a hydrophobic interaction chromatographic column; alpha-hemolysin prepared in this way retained its leukotoxic activity. Both hemolytic and leukotoxic activities co-purified at the same ratio and were inactivated to the same degree by heating at 56°C.

Lysis of leukocytes, if occurs in vivo, would enhance the chances of survival for an invading hemolytic *E. coli*. It has been demonstrated that other bacterial hemolysins also affect leukocyte functions such as phagocytosis (11), chemotaxis (12), and metabolic activation (13).

In order to check if some hidden factor influenced the leukocyte viability, a plot of Leukocyte viability vs Ratio of erythrocytes/leukocytes in radioactive labeled pellet was plotted (Figure 5). As could be foreseen, the two values appears totally uncorrelated, so proving that no hidden variables are responsible for the leukocyte viability variations.

All other investigated correlations between non viable/viable leukocytes ratio and nature of infection pathology, splenic and hepatic fixation, presence of epatomegalia or splenomegalia, presence of different systemic pathologies, medullary fixation were found not significant (P 0.01)

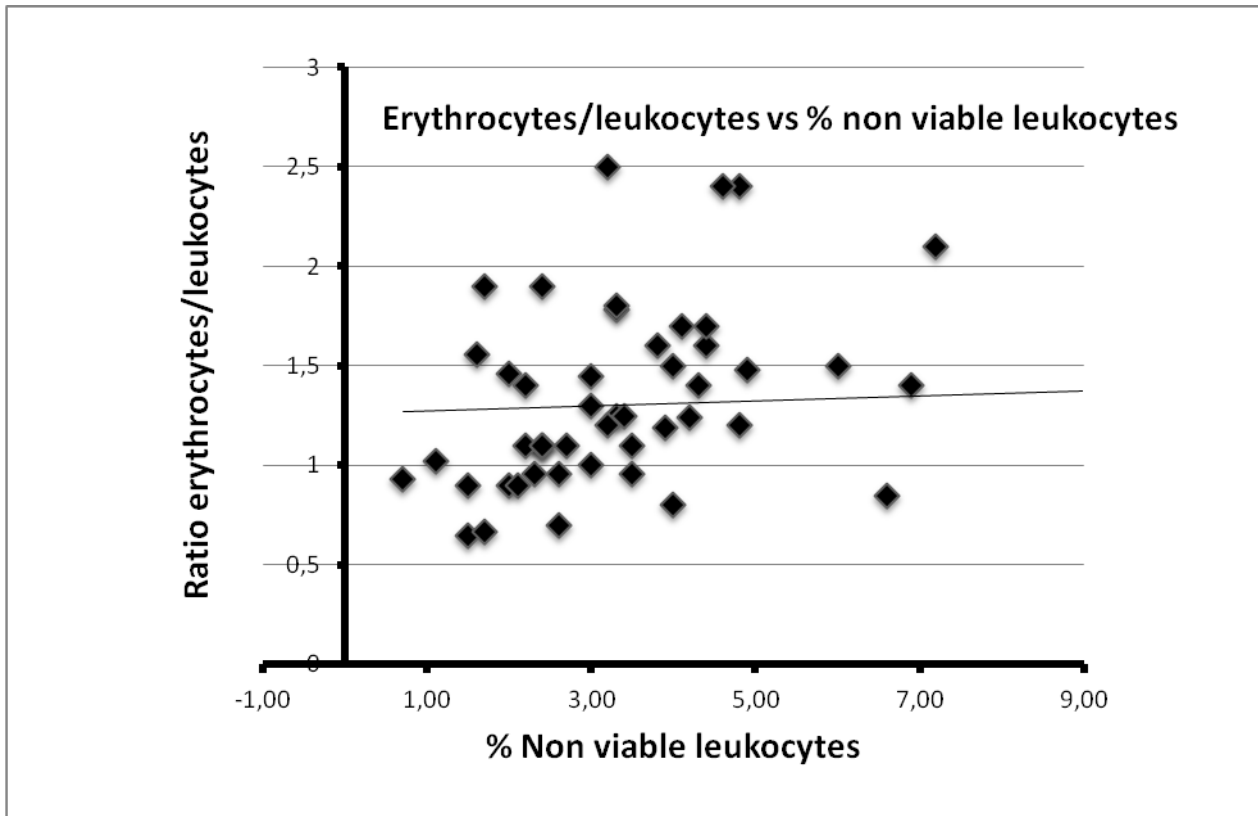


Figure 5

Discussion

Isolating or harvesting leukocytes from the whole blood requires a skilled operator to perform multiple isolation and manipulation steps, while ensuring maintenance of cell viability and product sterility. An uneven distribution of radioactivity within the cellular suspension can occur if the cellular pellet is inadequately re-suspended prior to the addition of ^{99m}Tc -HMPAO. Equally important is the poor cellular re-suspension before patient re-injection, leading to potential lung uptake due to microemboli. Measurements of leukocyte viability is an useful quality control protocol to identify eventual radiolabeling problems, assuring a reliable diagnostic procedure.

Neutrophils are the most abundant white blood cells, constituting 60-70% of the circulating leukocytes, and the most common cell type seen in the early stages of

acute inflammation. They are usually first responders to microbial infection, and are active in phagocytosis of bacteria. These cells are not able to renew their lysosome (used in digesting microbes) and die after having phagocytosed the pathogens (14); the life span of a circulating human neutrophil is about 5.4 days (15).

White blood cells play a role in the defense mechanisms and when this happens they need more energy; when active, they die off soon, typically in 24 hours, when passive they survive a few days longer, 7 to 10 days in tissues.

Neutrophils can also be detrimental in acute and chronic inflammatory diseases, as hyper-activation of these cells can cause tissue damage. Recently, the concept is emerging that neutrophils have functions beyond defense against pathogens. For example, they were shown to influence B cell activation and to inhibit T cell activation and proliferation (16,17).

Under homeostasis, the constant production of new neutrophils in the bone marrow needs to be counterbalanced by the clearance of the same amount of cells from the system. A reduced clearance and concurrent neutrophilia occurs in many chronic inflammatory diseases, and are often involved in the pathogenesis of these diseases. The organs implicated in neutrophil clearance are the same as those that house the marginated pool: the spleen, liver, and bone marrow.

After 24 h and 48 h from infusion of radio-labeled neutrophils, these organs contained the highest concentrations of label (18). As the very same organs are thought to contain marginated neutrophils, it is difficult to conclude whether the accumulation of label in these organs is a result of neutrophil destruction or migration, or extravasation followed by apoptosis.

Migration of radiolabeled neutrophils to peripheral tissues has indeed been shown to occur under non-homeostatic, inflammatory conditions (19), but during homeostasis, most tissues do not contain such cells, as whole-body imaging did not show any

evidence for accumulation of ex vivo-radiolabeled neutrophils in peripheral tissues (20).

The amount of free-flowing neutrophils in the bloodstream of an average, healthy human has been estimated to be $8\text{--}30 \times 10^9$ (21), to which the number of neutrophils in the marginated pool should be added. Consensus is that during homeostasis, the marginated pool comprises ~50% of all circulating neutrophils.

As above said, it has been demonstrated (9) that mononuclear leukocyte viability are detrimentally affected by *S. aureus* cells, an effect that can be modulated by blood or milk polymorphonuclear leukocytes.

Neutrophils are well known to migrate to sites of inflammation under the guidance of factors such as C5a, CXCL8 (IL-8), platelet-activating factor, and leukotriene B4 (22). Also, during acute inflammation, the total number of neutrophils in the bloodstream increases dramatically, so it is likely that inflammation influences the neutrophils half-life.

It has been demonstrated that ex vivo-labeled neutrophils showed an increased circulatory half-life compared with healthy volunteers in patients suffering from CML, sarcoidosis or liver cirrhosis, in splenectomized individuals, and when healthy volunteers received prednisolone (23,24). No difference was seen in patients suffering from rheumatoid arthritis and active inflammation or in healthy volunteers receiving G-CSF (25).

In one study labeled neutrophils of different patients with active infections had longer or shorter half-lives than healthy controls (23). Similarly, the half-lives of neutrophils from neutropenic patients differed, depending on whether neutropenia was drug-induced and whether the transfusion was autologous or from a healthy donor (23,24).

All these data demonstrate that inflammation can influence neutrophils half-lives and that the influence varies between or even within diseases. Although the importance of

the quantification of neutrophils half-lives and kinetics is evident, there is still a lot of uncertainty about neutrophils turnover in bone marrow and circulation. Difficulty in distinguishing between labeled cells residing in the blood and the ones released by the bone marrow is an important limitation.

From this point of view the use of leukocytes labeled in a mild way, in order to preserve as much as possible the natural viability, can be a powerful tool to obtain conclusive clinical data on neutrophils production rates in the bone marrow, on half-lives in the circulation, and on correlation between viability and infection.

The present work demonstrates that a wide viability span in leukocyte viability can be evidenced in a large cohort of patients. A slight relation was found with the patient age, reflecting the natural immunity cell senescence, but a much more significant correlation was found between loss of viability and degree of infection of the patient.

The information has not only a knowledge value, but could be conveniently used to find useful correlation with grade and presence of infection.

A pre-screening of leukocyte viability or a cellular histochemical investigation, in parallel with the radioactive labeling procedure, could be used to clarify the degree and temporal evolution of the viability decrease, and possibly to address the patients therapy by a more selective choice of antibiotics, particularly in the class of cumbersome and hard-to-heal infections.

Moreover, in patients in which radiolabeling and re-injection of autologous leukocytes has previously demonstrated the presence of an infection, a protocol could be proposed, in which the correlation of leukocyte viability and infection grade can be used to follow up the infection state, and check the antibiotic clinical efficacy with a simple, economic and non invasive blood test.

In the therapy of infection it should be of the utmost importance a simple test with the following characteristics:

- the possibility of differentiating bacterial from non-bacterial or non-specific inflammation
- a strong correlation between marker value and severity of bacterial infection
- a plasmatic marker reduction in case of effectiveness of antibiotic therapy
- treatment with steroids or non steroid anti inflammatory drugs does not alter the plasmatic marker value

This role was typically attributed to C reactive protein, but procalcitonin (PCT), pro-hormone of calcitonin, has been introduced in clinical practice; the measurement of the blood concentration of procalcitonin can be used as a marker of bacterial infection and correlates well with the degree of severity of the same (26). PCT, when compared to other markers such as IL-2, IL-6, IL -8, pcr and TNF-alpha, reveals the highest degree of sensitivity and specificity in the differentiation of patients with inflammatory response from those with sepsis of bacterial origin (27).

Neutrophil CD64, demonstrated using flow cytometry, can be used as a reliable diagnostic marker of infection and sepsis; this marker was found to possess a very high sensitivity (94.7%), but its specificity was poor (46.5%).

No significant differences were found between the diagnostic performance of CD64 and that of CRP: both have high sensitivity and low specificity (94.7% and 47.9%, respectively, for CRP). In contrast, procalcitonin had a better specificity (91%), but its sensitivity reached only 71.9% (28).

The most serious concern with antibiotic resistance is that some bacteria have become resistant to almost all of the easily available antibiotics; the best way to avoid this is to select an antimicrobial which targets the specific organism, rather than relying on a broad-spectrum antimicrobial. In all cases of serious and/or hidden infections probably the best solution for a prompt diagnosis and a reliable follow up

is the use of a multi-test response, in order to decrease diagnostic uncertainty and increase sensitivity.

In the present work it has been demonstrated that the infection grade in a large cohort of patients correlates with the viability parameter of patient leukocytes, a simple, economic and affordable test.

The leukocyte viability simple test could be used, together with the panel of the others described infection markers, to increase diagnostic reliability by raising the specificity, so clarifying the behaviour of the immune response of leukocyte in presence of antibiotic-resistant bacteria strains, a problem that is becoming more and more important, particularly in nosocomial infections.

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