Expanding the Frontiers of Dermatology: Skin Leucocytes Collected for Different Types of Studies in Immune deficient Subjects

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ABSTRACT

The skin is the largest organ of the body. It is the one entirely exposed to the environment. It has an outer avascular stratified, squamous epithelial layer with its appendages the epidermis, and a deep vascular connective tissue layer the dermis. The dermal blood capillaries exude fluid and leucocytes into the surrounding tissues in The exuded cells, and fluids are collected by blind ended lymphatic vessels starting in tissues and draining into major veins and the Cysterna Chyli. Lymphatic venules exist in all organs, except a few debatable sanctuaries. These vessels differ from blood capillaries and venules, by having discontinuous, scanty basal lamina, contoured by irregularly spaced, muscle fiber. The latter, suck in the extracellular fluid, by spontaneously contracting, in a fashion similar to cardiac muscle cells. The endothelial lining cells are separated by fenestrations always patent. Once the vessels are distended the fenestrations become covered by the endothelial cell extensions. To obtain pure leucocytes, it is enough to scrape superficially the epidermis, removing part of the keratinized dry layer, to open an epidermal window (EW). The superficial injury, induces an inflammatory reaction with both leucocyte components, the acute and chronic phases. Both types of responding cells (the granulocytes and the a-granulocytes) are collected on sterile slides or cover slips, for easy handling and use in many types of studies. These cells are studied for form, content, function, and can even be expanded in cultures. The EW has been and still is a very useful tool to identify the defect in patients complaining of poor immunity. In addition, it is used to evaluate the epidemiology of several diseases such as some infections, autoimmune disorders and even neoplasm. In this manuscript we are describing our experience with EW for more than 30 years, in patients suspected to be immune-deficient.

Keywords

Epidermal Window, Dermis leucocytes, Skin leucocytes.

Abbreviations

ESW: Epidermal Skin Window; EW: Epidermal Window; Fig: Figure; HIFBS: Heat Inactivated fetal Bovine Serum; penstrep: Penicillin Stroptomycin; RPMI: Rosewell Park Memorial Institute; WBC: White blood cells.

Introduction

Expanding the frontiers in any field must be the ambition of any specialty in any science or art. This report aims at using some morphologic components peculiar to the skin, which can become a source for testing the integrity of the body immune system under all circumstances.

This approach is built on the understanding of the histologic composition of the skin, which is characterized by a heavy network of lymphatic tissue, that may be a source of pure leucocytes. This source has been, so far, neglected by both the dermatologists, and hematologists. As a reminder the skin is composed of a vascular dermis covered by an avascular epidermis. In the dermis, the lymphatic component runs in parallel with the venous drainage system [1].

The intensive presence of the lymphatic vessels in the skin is central to safeguard this extensive surface area facing the external environment. The other organs with large surfaces in contact with the external environment are the gut, and the respiratory system. In short, lymphatic vessels start in any organ as capillaries. They are laced with a discontinuous network of collagen fibers that fixes
these vessels in place. In the skin, the lymphatic capillaries run close to the basement membrane underlying the basal layer of the epidermis. They have a special type of endothelium different from the blood capillaries lining cells [2].

This peculiar endothelium has the form of an oak leaf with cytoplasmic extensions as flaps, which overlap over one another, interconnecting with special types of junctional complexes, forming discontinuous button-like junctions, that run in a continuous line along the vessels in a zipper-like form [3]. These lymphatic capillary lining cell (LECs) with their flaps function mechanically as primary valves to control the movement of fluid. The openings among the flaps are fenestrations that facilitate the entry and exit of all that is present in the extracellular fluid whether small molecules in solution, or larger ones in suspension, or much larger bodies, especially if flexible and deformable, as all leucocytes are [4].

The junction proteins are typical of tight and adherent junctions, which allow movement of leucocytes in circulation, in and out the lymphatic lumen unharmed or changed. This remarkable superficial network makes the dermis, an important source of pure leucocytes. These cells can be collected by a scratch of the epidermis, referred to as an “Epidermal Window” (EW). Thus WBC can be collected pure, free of red blood cells and of any platelets which is an added advantage over any diagnostic biopsy of skin, or bone marrow. Besides, the leucocytes, are obtained live, functioning, and in high numbers, ready to be studied for different molecules within the cytoplasm or covering the cells. This method is far more efficient than obtaining them by purification of samples of blood, in which they are rather few, or getting them through more invasive procedures such as a biopsy/ or aspirate, of lymph nodes, or spleen as needed [5]. The technique we are reviving is the “Skin Window” (SW) described by Rebuck and Crowley [6].

Effectively our modified version of the “Skin Window” or more appropriately, the “Epidermal Window” allowed us, and still does, to study different types of leucocytes in patients suspected, clinically, to be immune deficient [7-11]. The obtained WBCs are studied morphologically, biochemically, and physiologically, in vivo and in vitro. In our hands, this test, for more than five decades, proved to be a very useful technique. It was, and still is, the most frequently used test that helped us reach a diagnosis in close to 99 out of every 100 patients studied for immune deficiency. It allowed us to realize that most of these immune deficient subjects, had a cryptic infection that either destroyed the leucocytes, or rendered them ineffective [12].

Microscopically in a minority of cases the defect was obvious such as an impressive reduction in the number of cytoplasmic granules (present in both categories the granulocytes and the agranulocytes [13] to poor adherence, lack of pseudopodia, and defective motility [14-17] resulting in inadequate engulfment, to lack of receptors [18] etc…

The leucocytes collected on the EW slides were extremely helpful in revealing the type of problem with the immune system. EW differentiated between patients with leucocyte constitutional or inborn errors (without determining the exact nature of the defect), and the patients who had leucocytes with poor performance secondary to their being invaded by infectious agents. Only in very few situations the infectious organism was present floating in the exuded serum and caught on the EW slides. In the greater majority of the tested subjects, the infectious microorganisms were, in fact, kept low in number, and within only a minor proportion of the types of leucocyte it invaded, which may explain missing them on a simple blood smear. Our results, using this test, uncovered a very important fact namely, that in regions endemic for certain infectious diseases, subjects carrying any such organisms, are far more numerous than ever suspected [19,20].

In this study, the controls were cases on whom the epidermal window was totally normal (Figure 1). Whenever EW failed to reveal the problem, which was the case of a very small percentage of the patients, we applied other immunologic techniques available in our laboratory to determine the exact defect. Every time the EW revealed the agent causing the subclinical infection, in any of the leucocyte types, we identified the microorganisms using one or more of several histologic techniques, using specific stains, that demonstrate the suspected infectious agent. Most of the times the histologic techniques were enough to determine the identity of the agent. Effectively once the foreign invader identified and the appropriate treatment used, the complaints of each patient disappeared and the subject resumed a normal life. Our experience with this test, led us to realize the importance of using it more frequently than any other, not only for testing patients with immune deficiency, but also in patients with certain autoimmune disorders [21,22] and, others with granulomatous diseases [23,24], and still others with certain types of neoplasm [25].

Figure 1: EW chronic response slide n2, 16hrs after scraping. Six monocytes in one field (vs 8 % on the peripheral blood smear of this patient). Every cell looks healthy, nucleus/cytoplasm ratio normal, adequately activated nuclear material, number of cytoplasmic vacuoles normal, cytoplasmic density normal, Normal EW chronic response. Modified Romanovsky stain (Wright/ Giemsa). Oil immersion lens (100 X mag).
Furthermore, it proved to be extremely useful in surveillance studies of some infections that affect leucocytes, and as important in uncovering the spread of several infectious agents and their vectors to geographic regions in which they never existed before [26-31]. In addition to reports on observations of climate change in some parts of the world such as observed in Europe [32-35].

Furthermore it proved to be extremely useful in surveillance studies of some infectious agents that attack leucocytes. It is as important in monitoring the spread of micro-organisms to regions in which they did not exist before, which seem to be the consequence of climate changes as noted lately in Europe [32-35], or catastrophic atmospheric instability (draughts and deluges) as happened in Ethiopia, or relocation of large populations (refugees) from one zone to another[36]. All nearly always lead to famines, precarious shelters, with tragic consequences on health [36-40]. Considering that EW requires no sophisticated equipment (needs only a microscope), with a minimum of material (slides, stains). it becomes particularly important in the epidemiologic investigation of the appearance, spread and reappearance of new and old microbes thought to have become either extinct or under strict control. Studying pure leucocytes and in high numbers, allowed us to avoid procedures far more sophisticated, and other costly techniques such as leucopherisis or else genetic studies which the majority of our patients, could not afford, a situation prevalent in most developing countries with or without political unrest.

The objective of the current study are two fold:
- To describe our technique in obtaining pure, live WBCs and in large numbers.
- To illustrate how leucocytes collected using the EW, can be used in, epidemiologic surveillance studies of many infectious diseases such as tuberculosis, brucellosis, leishmaniasis, tularemia, and Chaga's disease and many other infectious diseases.

Material and Methods

Each patient before we applied the test filled out a consent form, whereby we were allowed to use the data and the results in any study we run provided we used the initials only (not disclosing the identity of the patient). The next of kin signed for subjects who were under age. The patients who refused to sign the consent form are not included in this study. The EW is a sterile technique that consists of scraping the epidermis superficially over an area measuring about 20 x 50mm² (using a sterile no 4 surgical blade) to obtain"The Window".

When the scraped area becomes shiny humid (not a drop of blood is allowed), it is covered by a sterile microscope slide/cover slips (slide no1/cover slips no 1) to collect the cells reacting during the acute phase of the inflammatory response, namely after 4 hrs. Once the time is up, a new slide / cover slips no2, replace the first ones to collect the cells of the chronic response at hour 16 from the scraping time. Each slide/cover slips once removed, is/are placed in 50ml plastic sterile test tubes filled with either 95% or absolute ethanol for fixation and subsequent instead of staining or in similar test tubes filled with culture medium RPMI 1640, +Hepes buffer, plus 20-25% HIFBS + Antibiotics (usually Penstrep), that suits the growth of mammalian and non-mammalian cells (such as many types of microorganisms).

If after examining the stained cover slip we decide to isolate the observed microorganism, the other cover slip is put in culture in the appropriate medium. When the test was carried in the field (outside our laboratory) the test tubes with the harvested slide/cover slips were transported intact, sometimes for very long distances, provided the ones for culture are kept at any temperature above freezing and below 30°C. The fixed cells were stained using the appropriate stain that was indicated by the suspected diagnosis. The exhuded cells from the EW were studied microscopically using most often a modified Romanosky Wright / Giemsa stain [41a]. Part of the changes we introduced was to use supravital stains [41b], as well as in some instances (to confirm our diagnosis) immunofluorescence [41c].

In addition many times, the cells adherent to the slides were set up in cultures using the appropriate medium for the suspected infectious agent. This was necessary to obtain enough microorganisms to identify the species, subspecies, and strain of the isolated microorganism (whenever needed). Each slide/cover slip, was read evaluating the integrity of the collected cells (cytoplasm and its content such as cytoplasmic granules, vacuoles, presence of foreign bodies, as well as the nucleus, its size, shape content, presence of vacuoles, foreign bodies etc.). A differential was performed (as is done on a blood smear) within the granulocytes of the acute response, and the same for the cells collected for the As seen below, the acute response in patients with abnormal granulocytes is illustrated in the figure below.
us to use the cover slips with their adherent cells for more than one type of test and study. Finally we prolonged the harvesting time of the responding cells during the chronic phase of the response, to 20 hrs instead of 16 hrs, when the peripheral white blood cell count was around 2000/ml and to 22 hrs when it was less 1500/ml. These were very helpful modifications, That allowed us to use our results in more than one study.

**Results and Discussion**

The cooperation of the patients was exemplary, although many were illiterate they came for testing as often as we required. When they understood the cause of their problem (especially when it was an infection) they even brought members of their family or people living in the neighborhood for us to test, even when they had no similar complaints.

We reviewed the medical records, of 750 patients studied over 10 years, and in whom the diagnosis was reached using EW only. The cells obtained by the EW on these patients were always compared with controls of the same age group on whom the EW was totally normal (Figure 1).

The results obtained for the parameters mentioned for the acute and chronic responses of WBC by EW were always compared to the subject’s peripheral WBC on a blood smear assessed for all the parameters mentioned above. None of the tested patients had any clinically pathognomonic feature suggesting the cause of the immune-suppressed state. Diagnosis was revealed in 674 patients whose problems were successfully identified by EW.

Out of the 750 only 76 (10.1%) had an intrinsic defect. In the rest, about 70 patients, we successfully identified the deficiency using other techniques, available in our laboratory.

In the remaining 6 patients we failed to determine the cause of the problem and we referred them for genetic studies (only one or two could afford it). Effectively in the majority of the tested subjects, as mentioned above 674 patients had leucocytes invaded by one of several types of microorganisms without any typical clinical symptom (as mentioned before) the most salient complaints were excessive skin dryness with itching for no obvious reason, chronic poor immunity making the patient vulnerable mainly to viral infections and arthritis, in addition to vague muscle pain, low body weight in spite of a normal nutrition, frequent non bacterial bronchitis, chronic cough, frequent headache, repeated bouts of fever which would resolve spontaneously, for no obvious reason.

Lack of energy, and even sometimes depression with, or without different types of phobias etc...The signs in some cases were borderline to low white count with no other sign or complaint. The EW revealed in many of these patients anomalies in one type or the other of the granulocytes or the agranulocytes. The abnormal cells were activated, had flagrant pre-degenerative changes, a foamy cytoplasm, with vesicles sometimes even in the nucleus (Figure 3).

In the majority of cases, by far, though in a small proportion of the WBC, we detected intracellular live bodies in one type of leucocyte or the other. These intracellular organisms were any of many common infectious agents in our area such as *Mycobacteria, Toxoplasma, Aspergillus, Brucella, Leishmania*, (Figure 4) and less often, they were free in plasma, such as Rickettsia, and several other types of microorganisms (Figure 2).

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**Figure 3:** EW. Chronic response slide no 2 (16 hrs after scraping).

**Figure 4:** EW. Chronic response at 16 hrs, slide no2. Excessive response, Polymorphonucleated cell present at 16 hrs, it reached the “window” lesser than 4hrs after injury (slowed down motility). Highly vacuolated cytoplasm, no apparent granules, nucleus excessively segmented, with parts of the DNA condensed and inactivated. Asterix on degenerated dead cell. Arrow within a highly activated monocyte which has an excessively spread cytoplasm (2-2.5x its normal size), the cytoplasm is full of vacuoles and highly thinned down about to disintegrate. Shreds of fibrin coagulated on the slide. Cell with ballooned cytoplasm with vacuoles, and a totally pyknotic nucleus. Modified Romanovsky (Wright/Giemsa). Oil immersion lens (X100 mag).
In all such patients the correct diagnosis was reached in less than three days, when the appropriate therapy was started. At the end of treatment and in nearly every case the organism was irradiated and the symptoms and complaints disappeared. Rare was the case which needed a second course of treatment to eradicate the infection. Success of the treatment was proven by another EW after about 2-5 months after treatment, depending on the case. In our experience, we did not use EW, many of these patients, with chronic immune suppression, would have remained undiagnosed, and thus, threatened to develop more serious diseases such as neoplasms for example.

Furthermore it made us aware, that subclinical infections and subjects carrying infectious organisms are far more common than so far ever suspected. Finally EW helped confirm the diagnosis in some inflammatory conditions, as mentioned above, such as granulomatous diseases, autoimmune disorders, and certain types of cancer using tests for surface and intra-cytoplasmic markers on the related leucocytes.

Conclusion
To realize that the skin with its important lymphatic component can help diagnose a systemic infection is a real advantage. This remarkably easy access to the immune system becomes all the more important when the body’s reaction to certain infections is neither obvious nor typical. Furthermore access to pure and large number of leucocytes should be thought of, as a practical means, to detect infectious organisms by looking for the entire infectious agent, or for some of their surface or cytoplasmic markers, in and on leucocytes in any person. Simple and inexpensive tests are particularly important to apply nowadays on displaced subjects from one environment to a totally different one, even within the same area, as mentioned before, from south west to north east where the biotope is totally different, and from east to west, fleeing active wars.

Finally, the obvious serious climatic changes (whether droughts or deluges), often lead to conditions where a great portion of the society moves into precarious housing. Such adverse events are more often than not, associated with famines---, especially in the developing or underdeveloped countries, where food is scanty and malnutrition is very prevalent. All of this is invariably associated with poor health care, including nearly total absence of preventive medicine. The above situations, have never been as widespread around the world as they have been during the last few decades, and until the present times. In most of these situations the system most targeted is undoubtedly the immune system, which becomes under severe stress.

Here is apparently the value of a simple test like “EW”. No doubt, it becomes a very important, effective, easy to apply, inexpensive yet very efficient tool. EW will detect and thus allow control of most infections. It is a test not to neglect in national and international health institutions. It can be administered by any technician (who can be easily trained and at very low cost, and effort). The obtained slides from any surveillance study can be collected in the field and brought to a laboratory with no more than elementary facilities for staining and microscopy, and also, if possible, facilities for culturing cells. EW is certainly, cost effective, saving lives and sometimes entire families, and even generations. It is excessively important especially that it does limit the loss of personnel and of course it limits loss of work days. The alternative tests are more complicated and sophisticated tests (molecular and/or genetic techniques), the cost of which may not be affordable by many populations, especially when it is applied on a large scale in many countries around the world.

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References


