Improved Immunodiagnosis of Neutrophil Dysfunction in the Newborn and Infant

ALAN B. LOREN, M.D., PH.D., M. M. YOKOYAMA, M.D., PH.D., and EGIL FOSSLIEN, M.D.

Department of Surgery,
Loyola University Medical Center,
Maywood, IL 60153

and

Departments of Pathology and Microbiology,
University of Illinois Medical Center,
Chicago, IL 60680

ABSTRACT

An improved assay for the simultaneous assessment of phagocytic uptake (via Immunobeads®) and metabolic integrity (via nitroblue tetrazolium (NBT) dye reduction) was used to evaluate the neutrophil function in neonates, one year olds, and adults. The unique advantage of this assay is that it offers greater standardization since the beads are commercially available with a known quantity of immunoglobulin bound to their surface which allows for considerably less variation than particles opsonized in vivo.

Blood samples were collected from 20 full term healthy neonates, 20 healthy one year olds, and 20 healthy adults. The neutrophils were isolated, and phagocytic and killing function compared among the three groups. It was found that the neonates had a small but significant neutrophil dysfunction with respect to phagocytic and intracellular killing ability when compared to the one year olds and adults. Additionally, blood was collected and evaluated from five premature infants with varying degrees of stress. Their neutrophil dysfunction was much more pronounced.

Although it was previously thought that full term healthy neonates have no demonstrable neutrophil dysfunction unless stressed in some manner, a subtle dysfunction was identified even in unstressed neonates. It was, however, found to be more pronounced if the neonate was either premature or stressed in some manner. It is hoped that this assay, through its greater sensitivity and ease of standardization, will uncover subtle neutrophil dysfunctions in various disease states that as yet remain undiagnosed. The clinical significance of such subtle neutrophil dysfunctions is not yet known.

Introduction

Numerous methods have been described for analyzing neutrophil function and can be categorized according to the particular function being studied. Neutrophils have five basic functions: motility, recognition, ingestion, degranulation, and intracellular killing. A defect in any one of these functions is associated with an alteration in primary immune response...
of the host, resulting in an increased susceptibility to infection.

Two assays have been widely used clinically to measure the intracellular killing functions of the neutrophil,—the nitroblue tetrazolium (NBT) dye reduction test and the intraleukocyte killing test. It has been shown that neutrophils rapidly generate reducing power via the hexose monophosphate shunt in what is termed a metabolic burst.1-4,9 During this process if NBT is present, it will be reduced to formazaan and can be detected either by visual inspection or spectrophotometrically.3 The intraleukocytic killing test is performed by incubating viable bacteria with a suspension of lysed neutrophils and subsequent assessment of bacterial viability.5

An improved assay for the simultaneous assessment of phagocytic uptake (via Immunobeads®* and metabolic integrity (via NBT dye reduction) has been recently developed.14 The unique advantage of the Immunobead® assay is that it offers greater standardization since the beads are prepared with a known quantity of immunoglobulin bound to their surface which allows for considerably less variation than particles opsonized in vivo. The efficacy of this assay has been previously demonstrated in a study involving a small group of normal individuals and several patients with chronic granulomatous disease (CGD).14 In that study, all of the normal adult donors had neutrophils that engulfed the Immunobeads® and reduced the NBT dye. Additionally, the neutrophils from patients with CGD showed ingestion equal to or greater than that of normal neutrophils, but they were totally unable to reduce the NBT dye in all cases.

The purpose of this study was to use the newly developed Immunobead® assay in evaluating neutrophils as a function of age, and hopefully to settle the controversy as to whether or not neonatal neutrophils are less functional than adult neutrophils.

Materials and Methods

ISOLATION OF NEUTROPHILS

Approximately six ml of blood were collected in heparinized tubes from each of the donors. Twenty samples were from cord blood of full term neonates of uncomplicated pregnancy and delivery with APGAR scores of 7 or greater. Another 20 samples were from healthy children between the ages of one and two years. Finally, 20 samples were from healthy adults between the ages of 18 and 40 years. Additionally, two ml of venous blood were taken from five premature infants at three days of age.

The blood was diluted 2:1 with phosphate buffered saline (PBS) and, after mixing by gentle inversion, was layered on six ml of a double layer gradient of Ficoll-Hypaque solution. The bottom layer consisted of three ml of Ficoll-Hypaque solution (specific gravity 1.119) prepared by mixing 10 parts of 50 percent Hypaque* with 20 parts of 9 percent Ficoll 400†. The top layer consisted of three ml Ficoll-Paque® (specific gravity 1.007)‡. The solutions were layered in 16 x 100 mm round bottom plastic tubes † and centrifuged at 400 x g for 30 minutes at room temperature.6

After centrifugation, two cell zones were visible, the lower zone predominantly neutrophils and the upper zone predominantly lymphocytes. The neutrophil zone was removed by aspiration with a Pasteur pipet and placed in a conical plastic tube. † The suspension was then washed by adding 8 to 10 ml of phosphate buffered saline (PBS) and gently mixed. This was followed by centrifugation for 10 minutes at 350 x g at room temperature. The supernatant was then discarded, and the wash-

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* Bio-Rad Laboratories, Richmond, CA.
† Pharmacia Fine Chemical, Piscataway, NJ.
‡ Falcon Plastics, Los Angeles, CA.
ing procedure repeated two more times. After the third wash, the cells were resus-
pended in PBS with 10 percent fetal calf serum (PFS-FCS), and the cell count ad-
justed to $3 \times 10^6$ cells per ml.

**Preparation of Immunobeads in Nitroblue Tetrazolium**

Nitroblue tetrazolium§ was reconsti-
tuted in PBS by adding 6 mg NBT per ml of PBS and vortexed vigorously for one
minute followed by filtering of the insoluble residue. The NBT solution must be
shielded from light as much as possible, since NBT is a light labile compound and,
for optimal results, this solution was prepared fresh each day. Immunobeads®,
which are polyacrylamide beads coated with rabbit anti-human IgG antibody, IgG
class, were then suspended in the NBT solution at a concentration of 150 to 200 $\times$
$10^6$ beads per ml.

**Neutrophil Function Assay**

Two hundred $\mu l$ of the neutrophil sus-
pension were added to an equal volume of the Immunobead® suspension in 12 $\times$ 75
mm siliconized glass tubes. The tubes were then centrifuged for one minute at
160 x g at room temperature. The supernatant was drawn off to remove ex-
cess Immunobeads® and replaced with an equal volume of PBS-FCS. The tubes were then incubated for five minutes at
37°C. An incubation time of five minutes was chosen because it has been previ-
ously demonstrated that there is no sig-
nificant difference with respect to phago-
cytosis and NBT dye reduction when the incubation time is varied between five
and 30 minutes.14 After incubation, the pellet was gently resuspended and the
neutrophil function determined. This was
done by placing one drop of the cell sus-
pension on a microscope slide and cov-
ered with a glass coverslip. Two hundred
cells were then observed microscopically
at a magnification of 400 $\times$.

In this study, all neutrophils were
grouped into one of three categories; to-
tally functional neutrophils (TFN), par-
tially functional neutrophils (PFN), and af-
functional neutrophils (AFN). A TFN is a
cell that has the ability to ingest the Im-
munobeads®, indicating that the cell is
capable of phagocytosis in addition to be-
ning able to reduce the NBT dye (seen as a
color change from yellow to dark purple)
which indicates metabolic integrity and has
been shown to correlate with intra-
cellular killing ability.8*15 A PFN is defi-
ced as a cell capable of phagocytic func-
tion although it lacks the metabolic
integrity. The AFN is one that does not
ingest the beads at all, hence no assess-
ment of its metabolic integrity is feasible.

**Results**

The mean percentage of totally func-
tional neutrophils isolated from adults,
children one year of age, and neonates
was 81.0 percent, 80.8 percent, and 70.6
percent, respectively. The mean percent-
age of partially functional neutrophils was
10.2 percent, 9.6 percent, and 14.2 per-
cent, respectively. The mean percentage
of affunctional neutrophils was 8.8 per-
cent, 9.6 percent, and 15.2 percent, re-
spectively.

<table>
<thead>
<tr>
<th>Donor</th>
<th>TFN (Mean ± S.D.)</th>
<th>PFN (Mean ± S.D.)</th>
<th>AFN (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults (n=20)</td>
<td>81.0 ± 7.5</td>
<td>10.2 ± 4.3</td>
<td>8.8 ± 3.9</td>
</tr>
<tr>
<td>One year olds (n=20)</td>
<td>80.8 ± 3.4</td>
<td>9.6 ± 1.5</td>
<td>9.6 ± 4.2</td>
</tr>
<tr>
<td>Full term neonates (n=20)</td>
<td>70.6 ± 6.5</td>
<td>14.2 ± 5.8</td>
<td>15.2 ± 5.2</td>
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</tbody>
</table>

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§ Sigma, St. Louis, MO.
Additionally, the neutrophils of five preterm infants were isolated and evaluated. Two of the infants were 26 weeks gestational age. One had 68 percent TFN, 16 percent PFN, and 16 percent AFN. The other had 70 percent TFN, 12 percent PFN, and 18 percent AFN. Two 28 week gestational age infants were studied. One had 75 percent, 13 percent, and 12 percent TFN, PFN, and AFN, respectively. The other 28 week gestational age infant who was stressed by a complicated delivery with a low APGAR and resultant septicemia had 57 percent, 21 percent, and 22 percent, respectively. A 31 week gestational age infant who was considered stressed owing to septicemia had 53 percent, 22 percent, and 25 percent, respectively (table II).

**Discussion**

As with many *in vitro* systems, neutrophil function assays vary considerably in design. Included among the important variables are cell concentration, opsonic concentration, type of particle being ingested, and phagocyte:particle ratio. Despite these differences, results obtained by most investigators studying neonatal neutrophils have been reasonably consistent. One group of investigators has found that the neonatal neutrophil is equal to the adult neutrophil in phagocytic ability under "normal" conditions. However, a second group of investigations carried out under "stress" conditions concluded that the neonatal neutrophil is deficient in phagocytic ability when compared with that of the adult.

When intracellular killing function was investigated, similar results were obtained, i.e., studies under "normal" conditions showed no significant difference between neonatal and adult neutrophils while studies under "stress" conditions revealed a deficiency in intracellular killing function by the neonatal cells. It is our belief that there is indeed a deficiency in the neonatal neutrophil, and the defect is present at the level of both phagocytic ingestion and intracellular killing functions. In our study, the neonatal cells showed a significantly lower percentage of TFN's with a resultant increase in PFN's and AFN's. The main difference between our study and previous ones is that the present authors were able to uncover the defect under normal conditions while other investigators found the defect only under stress conditions. It is our contention that the controversy is related to the sensitivity of the assay.

An individual with a major neutrophil dysfunction becomes particularly susceptible to infections with *Staphylococcus aureus*, the enteric bacilli, and certain fungi (Candida and Aspergillus). Since these infections are not a problem in most neonates, it is likely that the neutrophil dysfunction, if present, is very slight. For this reason, the dysfunction could only be detected by a very sensitive assay. It is only when the neonate is "stressed" that the dysfunction becomes pronounced enough to be detected by less sensitive assays.

When the neutrophil function of presumably healthy one year olds was compared to that of adults, no significant difference was found. However, when

**Table II**

<table>
<thead>
<tr>
<th>Gestational Age</th>
<th>TFN (percent)</th>
<th>PFN (percent)</th>
<th>AFN (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>68</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>26</td>
<td>70</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>28*</td>
<td>57</td>
<td>21</td>
<td>22</td>
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<tr>
<td>28</td>
<td>75</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>31*</td>
<td>53</td>
<td>22</td>
<td>25</td>
</tr>
</tbody>
</table>

*Indicates the neonate was stressed.

TFN = Totally functional neutrophil, i.e. capable of phagocytosis and nitro blue tetrazolium (NBT) dye reduction.

PFN = Partially functional neutrophil, i.e. capable of phagocytosis, but not NBT dye reduction.

AFN = Afunctional neutrophil, i.e. incapable of phagocytosis hence no assessment of NBT dye reduction is feasible.
neutrophils from premature neonates were evaluated, the results were more dramatic. Owing to the small number of premature neonates in this study, the results are difficult to interpret completely, although the presence of a neutrophil dysfunction is obvious. In our study, the magnitude of the dysfunction does not appear to be related to gestational age; however, two of the neonates were stressed, which by itself seems to exacerbate the dysfunction. One of the stressed infants underwent a complicated delivery and, subsequently, had a low APGAR score and went on to develop infection. The other stressed infant was suffering from septicemia at the time of the evaluation. The stressed infants had a more pronounced neutrophil dysfunction, but they could not be equally compared to the others.

In conclusion then, our assay appears to be more sensitive than previous methods for evaluating neutrophil function, in addition to providing a simpler method for simultaneous evaluation of phagocytic and intracellular killing ability. Furthermore the existence of a subtle yet significant dysfunction has been demonstrated in neonatal neutrophils under normal conditions. Finally, our studies indicate that a stressed neonate will have an even more pronounced neutrophil dysfunction. It is hoped that this assay, through its greater sensitivity and ease of standardization, will uncover subtle neutrophil dysfunction in various disease states that as yet remain undiagnosed.

References