
Original Research Article

Bilirubin Crystals in Neutrophils: A Rare Occurance

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Abstract

Context: Neutrophils can demonstrate various inclusions upon examination in the stained peripheral blood smears. Hyperbilirubinemia is a common entity in the neonatal age group. Unusually, smears of children with hyperbilirubinemia can demonstrate bilirubin crystals in the white blood cells especially neutrophils.

Aims: To study the frequency of occurrence of these uncommon crystals in blood films of neonates which were examined during the work up for neonatal sepsis.

Settings and Design: This cross sectional study was conducted in a tertiary teaching hospital

Methods and Material: Neonates who were clinically suspected to have sepsis were evaluated for evidence of sepsis with the help of blood culture, blood cell counts and peripheral blood examination. Attention was paid to the presence of bilirubin crystals in the white blood cells and the same recorded.

Results: Smears examined from 174 neonates clinically suspected to have neonatal sepsis demonstrated the presence of these bilirubin crystals in 4.6% of the cases. Most of these cases were proven to have sepsis by blood culture.

Conclusions: Bilirubin crystals are a rare finding in smears of neonates having hyperbilirubinemia. They are frequently seen in septicemic neonates.

Keywords: Hyperbilirubinemia; Bilirubin Crystals; Neonates; Sepsis.

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Introduction

The peripheral smear examination happens to be an invaluable tool in the diagnostic armamentarium of the clinicians. The indications for peripheral blood film examination are well known. At times, serendipitously, various findings in the blood smear offer valuable diagnostic inputs. Morphological abnormalities of the formed elements help the clinician in formulating appropriate treatment plans.

At times, the formed elements show exotic cellular constituents which can surprise the unsuspecting pathologist examining the smear. One such entity happens to be the bilirubin crystals noted in the cytoplasm of the neutrophils. These crystals can be seen in a variety of conditions causing hyperbilirubinemia, especially in the neonatal age group.

This study aims to study the frequency of occurrence of these uncommon crystals in blood films of neonates which were examined during the work up for neonatal sepsis.

Materials and Methods

This study was undertaken in the department of Pathology over a period of two years from March 2004 to March 2006.

The blood samples collected from neonates who were clinically suspected to have sepsis and admitted to the neonatal intensive care unit of the hospital constituted the study material. Blood was collected under aseptic precautions in K₂ ethylene diamine tetraacetic acid (EDTA) vacutainers. The blood counts were determined using automated hematology analyzer. Peripheral blood smears were prepared from the anticoagulated blood and stained with Leishman stain. The smears were examined for morphological evidence of sepsis like left shift in the neutrophil series, increased band counts, toxic granules and vacuolations in the cytoplasm of neutrophils. Any other relevant morphological findings evident from the examination of the peripheral smear were recorded. The results of samples sent for blood cultures were recorded. All the relevant demographic and clinical details of the sick neonates were recorded from the case files.

Neonates who were admitted to the neonatal intensive care unit for other clinical conditions like respiratory distress of newborn, birth asphyxia etc., not clinically suspected to have sepsis were excluded from this study.

Results

During this study period, a total of 174 neonates who were clinically suspected to have sepsis were evaluated.

Based on the age of the baby, the clinically suspected neonatal sepsis group were further

categorized into early onset neonatal sepsis for neonates who were ≤ 3 days of age and late onset neonatal sepsis for those who were >3 days but ≤ 28 days of age. Of these 174 cases, the blood culture was positive in 112 cases. Of the remaining 62 cases, there was no growth in 54 cases and in 8 cases there were growth of contaminants.

In the present study 8 cases (4.6%) showed the presence of bilirubin crystals within the cytoplasm of the neutrophils. The number of crystals varied in each of the cell. These crystals were of various shapes like needle, rhomboid etc. These crystals were golden yellow colored refractile in nature. They were seen mainly in the cytoplasm of neutrophils (Figure 1). In all these cases the unconjugated bilirubin levels were elevated. The unconjugated bilirubin levels along with the corresponding blood culture reports are depicted in Table 1.

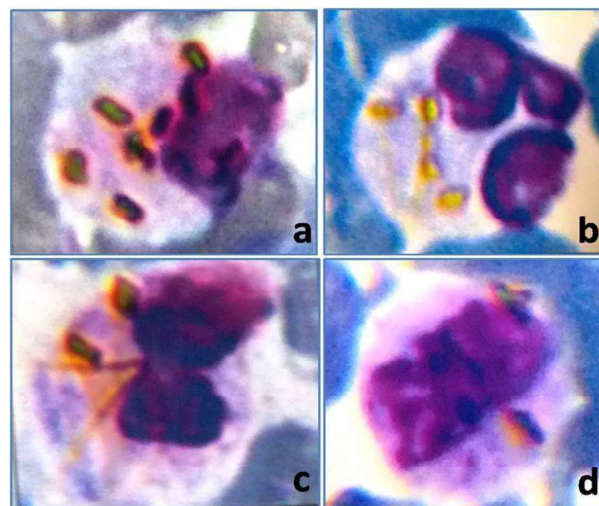


Fig. 1: Multiple rectangular (a,b,c,d), rhomboid (c) and needle shaped (c) golden yellow bilirubin crystals in neutrophils. 1000 X, Leishman stain

Table 1: Profile of cases showing bilirubin crystals in neutrophils

Age (days)	Sex	Unconjugated bilirubin ($\mu\text{mol/l}$)	Blood culture
3	Female	217.6	Coagulase negative staphylococcus
3	Male	212.7	Klebsiella pneumoniae
2	Male	212.5	Acinetobacter
3	Male	222.7	Coagulase negative staphylococcus
1	Male	221.0	No growth
2	Female	219.3	Escherichia coli
1	Male	214.2	Staphylococcus aureus
2	Female	207.4	Klebsiella pneumoniae

Fig. 1: Multiple rectangular (a,b,c,d), rhomboid (c) and needle shaped (c) golden yellow bilirubin crystals in neutrophils. 1000 X, Leishman stain

Discussion

The morphological examination of the peripheral blood film yields valuable clues in the management of various conditions. While some like the abnormal granules in neutrophils point to a specific disorder like Chediak Higashi syndrome, others like cytoplasmic vacuolations in the neutrophils could broadly indicate an underlying sepsis. One such unusual cytoplasmic inclusion in the white blood cells happens to be the bilirubin crystal.

Hyperbilirubinemia is a common clinical entity in the neonatal period. Hemolytic disease of the newborn and septicemia are some of the common causes of increased serum unconjugated bilirubin in the neonates.

The decreased solubility of unconjugated bilirubin in EDTA stored blood leads to its crystallization *in vitro*. A certain critical concentration of unconjugated bilirubin seems necessary for crystallization to occur. Crystals are only seen in smears from EDTA blood after a minimum incubation of 40 minutes at room temperature [1].

These crystals are yellow colored refractile, 0.8 to 2 μm sized and rhomboid, rounded, rectangular to irregular in shape. These crystals have been found in the cytoplasm of neutrophils and occasionally in band forms. Rarely may they be seen extracellularly. In the present study, all the crystals were seen intracellularly within the mature neutrophils [1,2].

Shenoi U et al in their study on the bilirubin crystals in the neutrophils in the neonates having hyperbilirubinemia noted that the proportion of crystal positive neutrophils was higher in septicemia and noted that these crystals were observed only when the serum unconjugated bilirubin was $>205.2\mu\text{mol/L}$. In the present study 8 cases of bilirubin crystals in neutrophils were noted. All these cases had an unconjugated bilirubin level $>205.2\mu\text{mol/L}$ which is in concordance with the study done by Shenoi et al. [3]. In these 8 cases, the blood culture was positive in 7 of them as depicted in Table 1.

Dange et al reported bilirubin crystals in two cases in a setting of unconjugated hyperbilirubinemia. Among the two, one child had history of Rh incompatibility while the second neonate was diagnosed to have sepsis. E coli was the organism isolated in the blood culture. In both these cases, blood smears prepared from EDTA anticoagulated blood showed the presence of the golden yellow refractile bilirubin crystals in the neutrophils. Such

crystals were not seen in other white blood cells. In the current study too, the presence of these crystals were restricted only in the neutrophils and no other white blood cell demonstrated these crystals. The authors studied the heel prick non anticoagulated blood smear in one of these cases and noted that such crystals were not seen [1]. No subsequent heel prick blood smears were examined in the current study.

High bilirubin levels have been associated with neutrophil dysfunction. However, it does not normally crystallize in the neutrophils. This is evidenced by absence of these crystals in fresh heel prick smears. In fact, only the sample collected in EDTA that is allowed to stand for 30-40 min show crystallization. Such crystals are not found in blood collected in other anticoagulants like heparin, oxalate or citrate.

Some authors have previously reported similar findings in neutrophils specifically in ethylene glycol tetra acetic acid (EGTA) anticoagulated blood. Sandoval et al. have noted that platelet-activating factor (PAF), a primary chemoattractant, simultaneously increases cytosolic free Ca^{2+} , intracellular pH, extracellular signal-regulated kinase (ERK) 1/2 and Akt/protein kinase B (PKB) phosphorylation and that the presence of EGTA reduced the intracellular alkalinization and ERK1/2 phosphorylation induced by PAF, apparently via store-operated calcium entry influx inhibition. Thus, it is possible that, by reducing calcium availability, it prevents alkalinization of the cytoplasm of the neutrophil leading to crystallization of bilirubin in acidic medium. This is also evident by the fact that bilirubin crystals may be seen in acidic urine. EDTA having a similar mechanism of action like EGTA reduces the calcium availability to the neutrophils and thus prevents alkalinization of cytoplasm leading to crystallization of bilirubin. However, why oxalate or citrates (calcium chelators) do not form crystals is not known [4].

Marwaha et al. studied blood smears of 118 hyperbilirubinemia neonates and 10 neonates with physiological jaundice. Most of the samples (107) were collected in EDTA and in 11 cases, capillary blood samples were used. Bilirubin crystals were in 63/107 (58.8%) smears examined in the neutrophils, band forms, metamyelocytes and myelocytes. Incidentally, these crystals were also occasionally noted in the monocytes too as well as extra cellularly. None of the 11 cases of non anticoagulated blood studied showed these crystals. The mean serum unconjugated bilirubin value was

significantly higher (293.8 $\mu\text{mol/l}$) in neonates with crystals in their smears as compared to those without crystals (242.2 $\mu\text{mol/l}$) [2]. This is similar to what has been seen in the current study and the study by Shenoi et al. [3]. In septicemic neonates the proportion of neutrophil-containing crystals was higher than in the case of other aetiological factors responsible for unconjugated hyperbilirubinemia. The present study too showed a similar pattern with most of the crystal positive cases belonging to culture positive septicemic neonates. Other case reports too highlight similar findings of bilirubin crystals in smears of septicemic neonates [5,6].

Some authors have also demonstrated that these crystals were birefringent, soluble in chloroform, and showed a positive reaction with Raia's indirect diazo reaction. Direct diazo reaction and tests for other pigments yielded negative results. The crystals could thus be identified as unconjugated bilirubin [7].

Similar to the bilirubin crystals, variable-sized, ill-defined, bright-green cytoplasmic inclusions have been seen in a subset of a patient's neutrophils admitted with septic shock. Similar neutrophilic inclusions have been reported in 2 patients with fatal acute hepatic failure just prior to death. The presence of such inclusions may serve as a prognostic indicator of impending death [8,9,10,11].

Conclusion

The finding of bilirubin crystals in neutrophils is specific to blood anticoagulated with calcium chelators, especially with EDTA and may be missed when other anticoagulants are used. Such a finding may be an indicator of bilirubin toxicity in unsuspected cases of hyperbilirubinemia due to several causes.

Further study needs to be done to see the cutoff level of indirect bilirubin at which these crystals start appearing in the cytoplasm of neutrophils.

The study re-emphasizes the importance of a careful examination of a blood film to avoid missing subtle but useful morphological findings in the era of automation in hematology.

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Conflict of Interest: None declared

Key Messages

Though uncommon, attention should be paid while examining blood films for the detection of bilirubin crystals in the neutrophils in septic neonates.

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