

Effect of ammonium chloride treatment on human polymorphonuclear leucocyte iodination

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SUMMARY A discontinuous gradient of Percoll was used to remove RBCs from polymorphonuclear leucocyte (PMN) preparations. The resulting red blood cell-free preparation was used to investigate the effect of ammonium chloride on the iodination response of human PMN. Treatment of PMN with ammonium chloride for five minutes at room temperature resulted in a statistically significant increase in the iodination response of both resting and stimulated PMN.

Many of the methods used for isolating human polymorphonuclear leucocytes (PMN) for functional studies result in a degree of red blood cell (RBC) contamination. A common solution to this problem is to remove the RBCs by differential lysis using ammonium chloride (NH_4Cl). RBC membranes are effectively permeable to NH_4Cl and cell lysis occurs due to the unbalanced osmotic pressure of their colloid content.¹

Both Boyle² and Shortman *et al*¹ have reported that NH_4Cl treatment does not harm lymphoid cells, although Shortman *et al*¹ warned of some damage occurring at 37°C. A recent report that NH_4Cl inhibits phagosome-lysosome fusion in murine macrophages³ suggests that this compound may not be totally without effect on phagocytic cells. Nevertheless, in spite of the widespread use of NH_4Cl to remove RBCs from PMN preparations, investigations of the effect of NH_4Cl on PMN have not been reported. One of the reasons for this may be the difficulty in separating the indirect influence of RBC removal from the direct effects of the NH_4Cl treatment.

In this communication this problem is eliminated by using a Percoll gradient to remove the RBCs from the PMN preparation prior to treatment with NH_4Cl . The direct effect of NH_4Cl treatment on the PMN can then be assessed in the absence of RBCs.

Material and methods

PREPARATION OF PMN

A RBC-free preparation of PMN was obtained using the method described by Phillips *et al* (submit-

ted for publication). Briefly, 8 ml of Ficoll-Isopaque prepared according to Boyum⁴) was layered under 10 ml of heparinised (10 IU/ml) human venous blood and centrifuged at 1500 RCF for 15 min. The upper layers containing plasma, mononuclear cells and Ficoll-Isopaque were removed and to the remaining RBC-PMN layer was added 3 ml 6% (wt/vol) Dextran T500 (Pharmacia, Sweden) and 10 ml phosphate-buffered saline (PBS) (0.1 M sodium chloride in 0.05 M phosphate buffer). This was mixed and allowed to settle for 30 min after which time the PMN rich supernatant was collected. Under 20 ml of this supernatant was layered 12 ml of 60% (vol/vol) Percoll solution and under that 15 ml 80% (vol/vol) Percoll solution (Percoll solution was prepared by mixing nine parts Percoll (Pharmacia) with one part 10 times concentrated PBS. This was then diluted to the appropriate concentration with single strength PBS). After centrifugation at 1200 RCF for 10 min the PMN layer, which was found at the 60%-80% interface, was collected and washed three times with 20 ml PBS by centrifugation.

The PMN preparation obtained in this way contains no mononuclear cells and minimal (<2%) RBC contamination.

AMMONIUM CHLORIDE (NH_4Cl) TREATMENT

In each experiment the PMN purified from 60 ml venous blood were divided into eight equal portions. At timed intervals, duplicate portions were centrifuged (800 RCF, 3 min) and resuspended in 10 ml Tris-buffered (pH 7.4), isotonic NH_4Cl (prepared as per Boyle² and passed through a 0.2 μm filter) at room temperature (25°C). PMN were treated with NH_4Cl for 5, 10 and 30 min. One set of

duplicates was resuspended in 10 ml PBS as an untreated control (0 min). The treatments were timed so that the different time intervals concluded together. All cells were then quickly washed twice with 10 ml PBS and immediately assayed for PMN iodinating activity using the method of Pereira *et al.*⁵ Heat-killed *Candida albicans* and *Staphylococcus aureus* were used at an optical density (540 nm) of 1.6. The final concentration of phorbol myristate acetate (PMA) (Sigma Chemical Company USA) was 1 $\mu\text{g/ml}$.

The viability of PMN after NH_4Cl treatment was confirmed using trypan blue exclusion.⁶ Results were statistically analysed using the Mann-Whitney U test.⁷

Results

The influence of NH_4Cl treatment on the iodinating ability of human PMN is seen in the Figure. Five minutes treatment was found to be sufficient to significantly ($p < 0.05$) increase the level of iodination by resting, *C albicans*- and PMA-stimulated PMN. The magnitude of this increase was directly related to the length of time in NH_4Cl . NH_4Cl treatment did not appear to significantly effect the *S aureus*-stimulated PMN iodination.

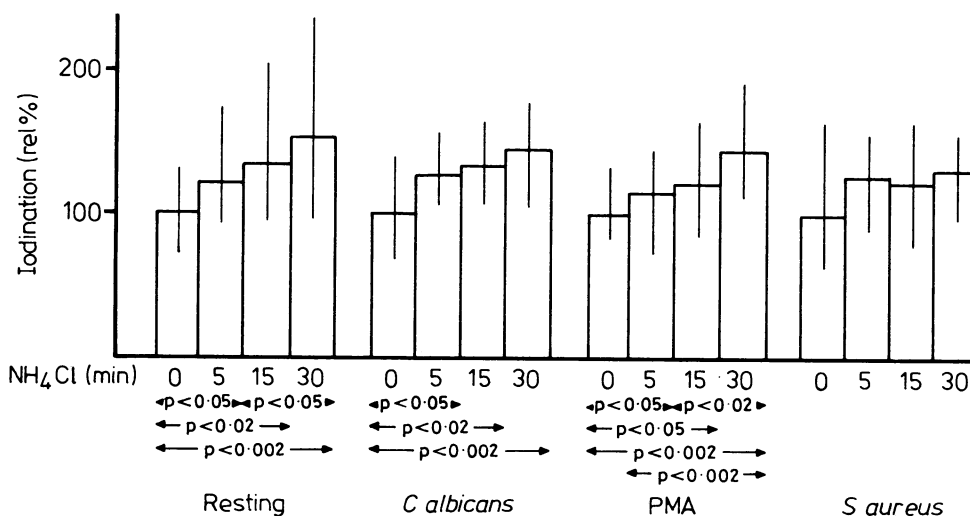
PMN treated with NH_4Cl retained a similar level of viability (as assessed by trypan blue exclusion) as the controls (>95%).

Discussion

In spite of the widespread use of NH_4Cl treatment to remove contaminating RBCs from PMN preparations, investigations of the effect of this compound on PMN have not, to our knowledge, been reported. One of the reasons for this may be the difficulty in separating the indirect effects of RBC lysis and removal from the direct effects of the NH_4Cl treatment on the PMN. This complication arises due to the fact that the control preparation, which has not been treated with NH_4Cl , will contain RBCs while the NH_4Cl treated preparation will not. This in itself may effect the results of PMN function assays. There is also the possibility that proteins and/or debris from the lysed RBCs could bind to the NH_4Cl treated PMN resulting in functional alterations that are not directly related to the NH_4Cl .

In this study we have eliminated these problems by using a Percoll gradient to remove the vast majority of RBCs from the PMN preparation prior to treatment with NH_4Cl . In this way the effect of NH_4Cl treatment on PMN is assessed in the essential absence of RBCs (<2% of total cells).

The results reported in this communication clearly confirm that NH_4Cl treatment does have a significant effect on the iodination response of the human PMN. At room temperature 5 min treatment was sufficient to increase significantly the iodination levels of both resting and stimulated cells. Many of



The effect of NH_4Cl treatment on PMN iodination. Shown are the mean and range of three experiments in sextuplicate. To allow the comparison of data from different experiments, results expressed relative to control (0 min) using the formula: $\text{Rel \%} = \text{cpm (data)} \times 100 / \text{mean cpm of control}$. In a representative experiment the mean raw data for iodination by the controls was: resting = 623 cpm; *C albicans*-stimulated = 2765 cpm; PMA-stimulated = 3255 cpm; *S aureus*-stimulated = 2349 cpm

the reports in the literature use twice this length of time at either 4°C,^{1,8} room temperature^{9,10} or 37°C¹¹ to remove RBCs. One author even suggests an NH₄Cl treatment of 30 min, or more, at room temperature for removing RBCs.¹²

Treatment at temperatures other than room temperature or the effects of NH₄Cl on other PMN functional parameters were not investigated. Five min was the shortest time tested because of technical difficulties in removing the NH₄Cl in less than this time.

While this effect of NH₄Cl is significant and reproducible, the mechanism involved is not clear. The NH₄Cl is removed from the cells before they are assayed for iodinating activity. The observed increase in iodination is therefore not due to an influence of NH₄Cl on the assay but must reflect an alteration in the activity of the PMN. Clearly further investigation is required to establish the molecular basis of this effect and to determine if other PMN functions are also influenced. In the meantime, when using NH₄Cl treatment to remove RBCs from PMN preparations, we suggest that due consideration should be given to the possible influence of this compound.

References

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