

## *Entamoeba histolytica*: elevated nitroblue tetrazolium reduction activity in polymorphs during amoebic liver abscess

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### Abstract

Peripheral blood polymorphonuclear leucocytes (PMN) from patients with invasive amoebiasis, i.e. amoebic liver abscess (ALA) and acute amoebic dysentery, showed marked elevation of nitroblue tetrazolium dye (NBT) reduction. This dramatic change was not observed in PMN from patients with non-invasive amoebiasis, i.e. non-suppurative hepatic amoebiasis, or in asymptomatic *Entamoeba histolytica* cyst passers. A small number (12%) of patients with viral hepatitis displayed increased NBT reduction. 10 to 12 days after recovery following treatment, the majority (75%) of ALA patients failed to show increased NBT reduction. Our results suggest that the PMN-NBT reduction test could be useful as an aid to the diagnosis of ALA.

### Introduction

A small proportion amongst the 15% or more of those in endemic regions who have *Entamoeba histolytica* in the colon, develop invasive diseases in the liver. The pathogenesis of amoebic liver abscess (ALA) remains unknown. Polymorphonuclear leucocytes (PMN) are present in lesions of ALA (RAVI *et al.*, 1974; TANDON *et al.*, 1975) and could contribute to tissue damage. Following PMN membrane perturbation, these leucocytes undergo a burst of respiratory activity, as a result of the activation of a non-mitochondrial respiratory chain (NADPH oxidase) (ROSSI *et al.*, 1976). This leads to reduction of oxygen and production of oxygen radicals which, although designed to kill microorganisms, may also damage tissues. The burst of oxygen-dependent respiratory activity in PMN can be measured by any one of a number of methods. These include the measurement of nitroblue tetrazolium (NBT) reduction (SEGAL & PETERS, 1975; GIFFORD & MALAWISTA, 1970). NBT reduction is related to the hexose-monophosphate shunt, known to be closely linked to NADPH oxidase activity (BORREGAARD, 1984).

In the present study, the NBT test was used to identify stimulated PMN in patients with invasive amoebiasis and acute inflammatory liver disease due to viral hepatitis.

### Materials and Methods

#### Subjects

The patients with amoebic liver abscess, acute amoebic dysentery, non-suppurative hepatic amoebiasis or viral hepatitis, which included acute fulminant (15), subacute hepatic failure (3) and acute viral hepatitis (AVH) (16), were taken from the wards of the Department of Gastroenterology, Rajgarhia Liver Research Unit, All-India Institute of Medical Sciences, New Delhi. Asymptomatic *E. histolytica* cyst passers were selected from gastroenterology out-patient clinic and normal healthy controls were chosen from departmental staff with no evidence of any active or recent bacterial or parasitic infections. The diagnosis of different conditions of amoebiasis was made according to standard criteria (WHO, 1969) and the diagnosis of different subgroups of viral hepatitis was based on accepted clinical, biochemical and histological criteria (TANDON *et al.*, 1983; TREY & DAVISON, 1970; TANDON *et al.*, 1982).

In patients with amoebic liver abscess, pus was aspirated for management and to confirm diagnosis and the patients were treated with metronidazole 2.4 g daily for 7 days. From 20 of these 29 patients, a second sample was drawn 10 to 12 days after clinical recovery and the NBT test was repeated. The remaining patients did not attend follow up.

#### NBT reduction test

The NBT test was performed essentially as described by HELLMUM (1977). 2 ml of blood were drawn from the antecubital vein and mixed with 20 i u of preservative-free heparin (10 i u/ml). Within 30 min, 50 µl of this blood were mixed with 50 µl of 0.05% NBT in phosphate buffered saline, pH 7.2 (PBS) and the mixture incubated at 37°C in a water bath for 15 min with occasional shaking. After incubation, the tube was kept at room temperature for 15 min. A thin smear of resuspended blood-NBT mixture was prepared on a glass slide and allowed to air-dry at room temperature. The slide was fixed with methanol for 15 min, then washed and air-dried. A count of NBT-positive PMN was made and these were expressed as a percentage of the total PMN. The test was run in duplicate. 200 PMN were counted in each test and classified as either NBT-negative, i.e. without visible deposits of formazan, or NBT-positive when containing deposits of formazan.

Based on the results obtained with 40 healthy controls, 20% NBT-positive PMN was taken as the upper normal limit. Scores above this limit indicated an NBT-positive test.

### Results

Patients with ALA showed a marked increase in the number of PMN able to reduce NBT, when compared to PMNs of healthy control subjects ( $P < 0.001$ ); the mean NBT score in the former group was 75% and, in the latter, 9% (Table 1). All ALA subjects gave a positive result (score >20%), while all the normal, healthy subjects were negative (Table 1).

In 20 of the 29 ALA subjects, the NBT test was repeated after clinical recovery. The mean NBT score had then dropped to 16% and only 5 out of the 20 were positive ( $P < 0.001$ ) (Table 2).

All 3 subjects with acute amoebic dysentery showed a high NBT score and a positive NBT test (Table 1). However, the non-suppurative hepatic amoebiasis group showed NBT scores comparable to the healthy subjects and none had a positive NBT test. The *E.*

Table 1—NBT score in different groups of subjects

Groups	No. tested	NBT score (%) mean $\pm$ S.D.	Range	Number and percent positive
Amoebic liver abscess	29	75 $\pm$ 17	38-99	29 (100%)
Acute amoebic dysentery	3	55 $\pm$ 16	33-70	3 (100%)
Non-suppurative hepatic amoebiasis	11	10 $\pm$ 4	4-18	0 (0%)
<i>E. histolytica</i> cyst passers	12	21 $\pm$ 8	10-38	5 (42%)
Viral hepatitis	34	10 $\pm$ 9	2-38	4 (12%)
Healthy controls	40	9 $\pm$ 5	2-20	(0%)

Table 2—NBT test in acute and recovery stages of ALA (n = 20)\*

	Acute	Recovered
NBT score (%), mean $\pm$ S.D.	75 $\pm$ 17	16 $\pm$ 8
Positive tests, number and percentage	20 (100%)	5 (25%)

\*Values are presented for only 20 patients with ALA in whom the NBT test was carried out during acute and recovery stages.

*histolytica* cyst passers also had low NBT scores compared to those with ALA ( $P < 0.001$ ) (Table 1).

PMN from a group of patients with viral hepatitis had an NBT score similar to that of healthy subjects and only 12% of these patients gave positive NBT tests (Table 1).

Comparative NBT-scores in groups with positive NBT tests are presented in the Figure.

### Discussion

Peripheral blood PMNs from subjects with invasive amoebiasis (amoebic liver abscess and acute amoebic dysentery) showed markedly elevated NBT reduction activity compared with those from healthy controls or from subjects with non-invasive forms of the disease (non-suppurative hepatic amoebiasis and *E. histolytica* cyst passers). This finding may have important implications in the pathogenesis of invasive amoebiasis since previously it has been demonstrated that there is PMN infiltration in lesions of ALA (RAVI *et al.*, 1974; TANDON *et al.*, 1975). The high NBT score in patients with ALA shows that a larger proportion of the neutrophil population in these subjects is stimulated to produce a family of toxic oxygen products such as superoxide anion,  $H_2O_2$ , hydroxyl radical and others (BORREGAARD, 1984; HOLMES *et al.*, 1967). In this manner these cells could contribute significantly to the tissue damage in ALA.

It is not surprising to find that PMN are stimulated in this condition. *E. histolytica* contains polyclonal T cell activators (DIAMANTSTEIN *et al.*, 1980; 1981). Such stimulation of T cells could lead to the release of a range of lymphokines (e.g. interleukin-2, interferon, leucocyte inhibitory factor, colony stimulating factor, etc.), all of which induce a respiratory burst in PMN (FERRANTE & RENCIS, 1984; PAK *et al.*, 1980; FERRANTE & MOCATTA, 1984; WEISBART *et al.*, 1985).

NBT SCORE IN DIFFERENT GROUPS

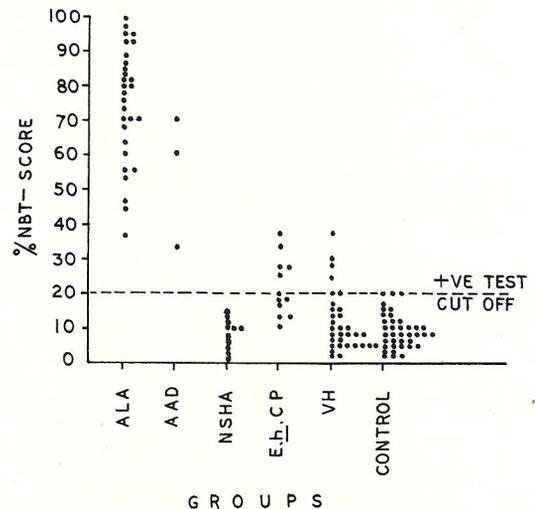


Fig. NBT scores of different groups of subjects. ALA, amoebic liver abscess; AAD, acute amoebic dysentery; NSHA, non-suppurative hepatic amoebiasis; *E. h. CP*, *Entamoeba histolytica* cyst passers; VH, viral hepatitis.

While this is a feasible means by which the PMN in ALA are stimulated, other mechanisms could also contribute. These include PMN stimulation by products generated from complement activation (e.g. C5a), especially as *E. histolytica* can activate complement by the alternative pathway (HULDT *et al.*, 1979). As this level of neutrophil NBT reduction activity was markedly reduced following clinical recovery, it is most likely that *E. histolytica* was contributing by generating complement factors acting on neutrophils, so that the effect was short lived. If lymphocyte activation was contributing significantly by releasing lymphokines, the effect would be expected to last longer.

HELLUM (1977) observed a frequent association between a positive PMN-NBT test and acute viral hepatitis, but not acute viral infections. In contrast, we found that patients with AVH had a mean NBT score very similar to that of the healthy subjects. Only 4 of 34 (12%) of these patients had a positive NBT test. This group of patients consisted of 16 with sporadic acute hepatitis, 15 with fulminant hepatitis

and 3 with subacute hepatitis. Of the 4 patients with a positive PMN-NBT test, 3 were in the fulminant hepatitis group and 1 in the subacute hepatitis group. 6 of 34 were positive for hepatitis B surface antigen (HBsAg) and only one of these 6 was NBT-positive. The reasons for differences between HELLUM's (1977) results and ours are not clear. In children with HBsAg-positive chronic active hepatitis, VIERUCCI *et al.* (1983) observed an increase in oxidative metabolism of resting neutrophils, demonstrated by the increase of superoxide-generating unstimulated cells, whereas in our study the NBT test in AVH seems to be least related to the presence of HBsAg.

Since the elevated NBT reduction activity of PMN was associated with acute ALA, the test may have some diagnostic value.

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