Evaluation of neutrophil functions in obstructive jaundice patients

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ABSTRACT

Jaundiced surgical patients have a high incidence of post-operative complications and sepsis is a major factor in the high mortality and morbidity of obstructive jaundice patients after surgery. Cachexia and immune suppression have been shown to be involved in this post-operative complications. Since neutrophils dysfunction is recognized in recurrent infections in general, the aim of the study was to evaluate the functional status of neutrophils in jaundiced patients. Phagocytosis, bactericidal activity, mitogenic stimulation of T-lymphocytes and myeloperoxidase activity were measured in the neutrophils isolated from obstructive jaundice patients. Phagocytic and bactericidal activities of neutrophils were significantly low in obstructive jaundice patients. The number of cells stained for myeloperoxidase was significantly low in obstructive jaundice patients. There was a high level of stimulation and proliferation exhibited by lymphocytes when challenged by conconavalin-A and lipopolysaccharide in vitro. The functional impairment of neutrophils may be responsible for the immunity alteration in obstructive jaundice patients and this may be responsible for the post-operative complications in obstructive jaundiced patients.

Key Words: Obstructive jaundice, Phagocytosis, Bactericidal activity, T-cells; stimulation, Myeloperoxidase.

ÖZET

Tıkanma sarılıklı hastalarda nötrofil işlevleri

Sarılıklı cerrahi hastaları sık cerrahi sonrası komplikasyonlarla karşılaşırlar ve sepsis, tıkanma sarılıklı hastalarda cerrahi sonrası mortalite ve morbiditede ana faktördür. Bu cerrahi sonrası komplikasyonlarda kaşeksi ve immünsüpresyonda rol oynar. Tekrarlayan infeksiyonlarda nötrofil disfonksiyonu gözlendiğinden, bu çalışmanın amacı sarılıklı hastalarda nötrofil işlevlerine bakılmasıdır. Bunun için sarılıklı hastalardan elde edilen nötrofillerde fagositoz, bakterisidal aktivite, T-lenfositlerin mitojenik uyarımı ve miyeloperoksidaz aktivitesi ölçülmüştür. Sonuçta tıkanma sarılıklı hastalarda nötrofillerin fagositik ve bakterisidal aktiviteleri belirgin derecede düşük bulunmuştur. Tıkanma sarılıklı hastalarda miyeloperoksidaz alan hücrelerin sayısında da düşüklük görülmüştür. İn vitro konkonavalin-A ve lipopolisakkarit ile karşılaşan lenfositlerde de yüksek düzeyli stimülasyon ve proliferasyon görülmüştür. Nötrofillerin işlevsel bozuklukları tıkanma sarılıklı hastaların immünitelerinde bozulmadan sorumlu olabilir ve bu da cerrahi sonrası komplikasyonları arttırabilir.

Anahtar Kelimeler: Tıkanma sarılığı, Fagositoz, Bakterisidal etki, T-hücre; uyarımı, Miyeloperoksidaz.

INTRODUCTION

Many evidences support an immunological pathogenesis in the development of jaundice due to extra hepatic obstruction[1]. Jaundiced surgical patients have a high incidence of post-operative complications[2]. Many causative factors have been identified including cachexia and immune suppression[3]. Sepsis is a major factor in the high mortality and morbidity after surgery for obstructive jaundice.

Ishida et al have reported that nutritional and immunological status of patients with malignant obstructive jaundice were severely depressed[4]. An anti-inflammatory response in patients with obstructive jaundice caused by biliary malignancy has been reported by Kimura et al[5]. Increased production of tumour necrosis factor (TNF) and interleukin-6 (IL-6) has been noted in animals models of obstructive jaundice[6].

Neutrophils or polymorphonuclear leukocytes (PMNL) are "dedicated professional phagocytes". They constitute about half of the circulating leukocytes in children and about two thirds in adults. They are capable of searching out and killing invading microorganism in different sites. Following invasion on the neutrophils show the following sequential activities:

a. Adherence to vessel wall[7],

b. Unidirectional migration towards invasion site[8],

c. Phagocytosis of microorganism usually opsonised or coated with IgG and C3b[9],

d. Intracellular killing and digestion of the ingested microbes associated with "degranulation" and "respiratory burst" of the neutrophils[10].

Neutrophilic dysfunction is characterized by recurrent infections with pyogenic bacterial (eg) staphylococci and gram-negative bacilli. This would happen when neutrophils are quantitatively reduced in the peripheral blood. This would also happen if opsonization is impaired due to IgG hypo gammaglobulinemia or complement deficiency. With normal neutrophil count and normal opsonization, intrinsic defects in one or more of the four above activities would account for recurrent pyogenic infections. Qualitative deficit of neutrophils is usually measured in vitro.

Several data indicate that in jaundiced patients activation of T-cells by specific antigens may occur and in this respect evidence of staphylococci and *Vibrio cholerae* infections, seem to suggest bacterial pathogenesis in this disease[11,12].

The first line defense carried out by the organism, the inflammatory response happens to the support of phagocytic cells. The PMNL and mononuclear cells (MØ) are important cell line acting as the first defense of the organism against bacterial agents. PMNL and MØ represent an important effector function in the control of bacterial infections[13]. The relative contribution to the host defense is frequently assessed by means of microbiological assays.

Despite a vast array of studies done on the immunocompetence of cells from blood, liver and gall bladder in obstructive jaundice cases, only a limited evidence is available regarding the functional aspects of lymphocytes, this study was planned to evaluate the functional status of lymphocytes in obstructive jaundice patients.

MATERIALS and METHODS

Patient Group

Patients admitted in the Department of Surgical Gastroenterology and Proctology with the complications of jaundice were diagnosed by various diagnostic methods. The diagnostic methods applied were abdominal radiography, oral cholecystography and abdominal ultrasonography.

The jaundiced patients with confirmed obstruction due to gall stone either in the gall bladder or in the common bile duct were

considered as group I; jaundiced patients with cancer in the head of pancreas are carcinoma in the periampullary region were considered as group II; jaundiced patients with stricture in the common bile duct were taken as group III; age and sex matched normal volunteers were utilized in this study for the comparison of results.

The protocol was approved by the ethical committee of Stanley Medical College and Hospital, Chennai and the patients gave their informed consent. The patients were of both sexes and in the age group of 30-35 years.

Blood samples were collected from obstructive jaundice patients who were to undergo surgery in a couple of days.

Methods

Isolation of lymphocytes: The method adopted for the isolation of lymphocytes was that of Boyum by using Ficoll-Hypague Medium[14].

Isolation of polymorphonuclear leukocytes (PMNL): To isolate PMNL, after Ficoll Hypague density gradient centrifugation, cell pellets were mixed with 6% dextran solution. After sedimentation for 1 hr at 37°C, the PMNL rich supernatant was collected and centrifuged at 400 g for 10 min.

Isolation of mononuclear cells (MØ): Fetal calf serum coated tissue culture flask were used for the isolation of MØ as described by Kumagai et al[15].

Study of *Candida* phagocytosis in vitro: The method of Mandell and Hook was adopted for the determination of the capacity of neutrophils to engulf *Candida albicans* as a measure of phagocytosis[16].

The test relies on the uptake of heat killed *C. albicans* by phagocytes over a period of time. The intracellular *Candida* stains intensely with Leishman's stain and can be identified and counted inside the neutrophils.

Assessment of bactericidal activity: Neutrophils separated from peripheral blood are challenged in vitro by a number of bacteria, at the approximate cell bacterial ratio of 1:1. Phagocytosed bacteria are separated from unphagocytised one by differential centrifugation. Their survival is assessed from their capacity to grow quantitatively on nutrient agar medium[16].

Assessment of antibacterial activity: The assay was performed according to Antonaci et al[17]. Briefly, 10^4 *Salmonella typhi* bacteria (target cells) were mixed with MØ depleted PBM cells (effector cells) at different effector/target (E/T) ratios and incubated at 37°C for 2 hrs. Aliquots of these mixtures were then plated on dishes containing agar trytose. After overnight incubation, colony forming units (cfu) were counted and the percentage of antibacterial activity was calculated as follows:

$$\frac{100 \times \text{number of cfu in experimental tubes}}{\text{no. of cfu in control tubes without PBL}}$$

Determination of Myeloperoxidase Activity by in Neutrophils

a. By o-toluidine staining method: The method is based on the reaction between peroxidase containing granulocytes and o-toluidine in which o-toluidine is oxidized and stained with Leishman stain to appear as yellow brown precipitate inside the cells and the stained cells are counted under oil immersion microscope[18].

b. By NBT staining method: It is the measure of myeloperoxidase activity in the neutrophils. Myeloperoxidase oxidizes NBT, and the oxidized compound reacts with Wright stain and appear as blue coloured bodies inside the cells[19].

Determination of Mitogenic Stimulation of Lymphocytes

The method followed for this determination was based on the report of Kang et al[20]. When lymphocytes are challenged with antigenic stimuli in the form of mitogens they are

activated and proliferate. When radio active thymidine is supplemented in the culture medium it is incorporated into the DNA synthesized. The radio active counting measured in the lymphocytes after separating the unincorporated lymphocytes is a measure of T-cell activation and proliferation.

The values were subjected to analyses of normal tests of significance. RA Fishers null hypotheses was adopted to find out the significance of variations between the normal subjects and obstructive jaundice patients[21]. For statistical analyses groups 1, 2, 3 were compared with group 4.

RESULTS

The level of phagocytosis exerted by PMNL and MØ were significantly reduced in all the three groups of obstructive jaundice patients studied when compared to those of normal controls ($p< 0.01$) (Figure 1). The difference between patients of different etiology were not significant. Figure 2 represents the level of bacterial killing in control subjects and OBJ patients. There was a significant reduction in the level of bacterial killing in group II patients.

Table 1 shows the percentage of cells stained for myeloperoxidase activity by the reducing agents o-toluidine and nitroblue tetrazoleum. The number of cells stained for myeloperoxidase was significantly low in obstructive jaundice patients when compared to that of normal subjects.

In all the groups of obstructive jaundice patients studied the antibacterial activity against *S. typhi* was significantly depressed at all E/T ratios evaluated ($p< 0.001$) even though more significant reduction was found at the lowest ratios (i.e., 0.7:1.0 and 0.3:1.0) (Figure 3). Again the difference between the three groups of patients were not significant except group III.

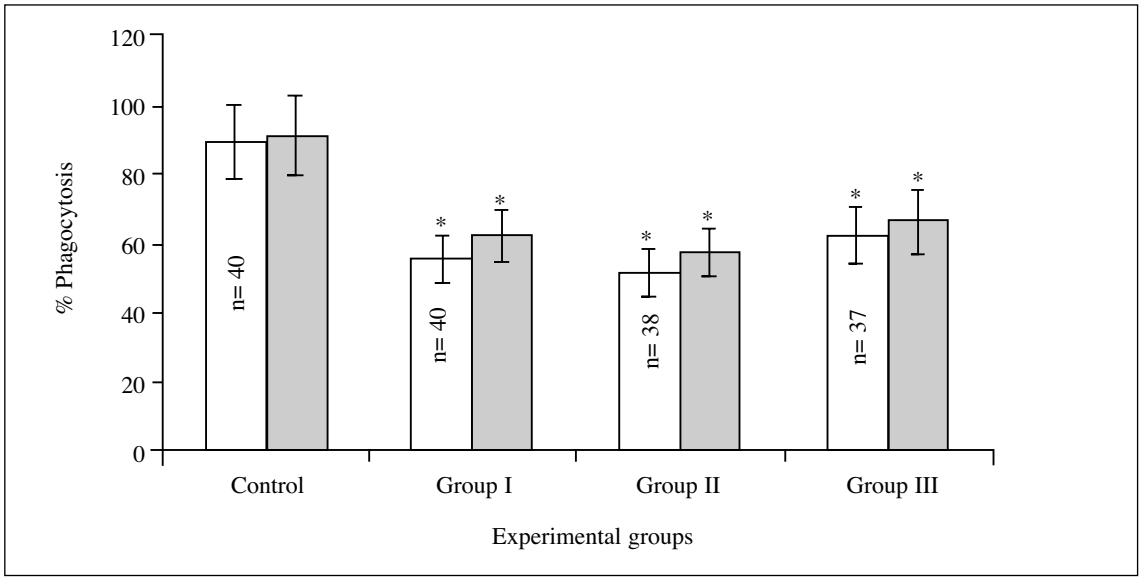

Figure 1. Phagocytosis exerted by PMNL (□) and MØ (▩) in normal controls and in OBJ patients. 0.2 mL *Candida albicans* (2 x 10^2 cells), 0.3 mL phosphate buffered saline, 0.1 mL pooled human serum and 0.1 mL Hank' solution were incubated at 37°C for 30 minutes. The smear prepared with a drop of this suspension was examined through oil immersion microscope after staining with Leishman stain and the number of cells with engulfed *Candida* were counted. The values were expressed as percentage of stained cells in the total cell population. The values are mean of ± SD for "n" individual experiments. Statistically significant difference are expressed as $p< 0.01$ when compared to control subjects.

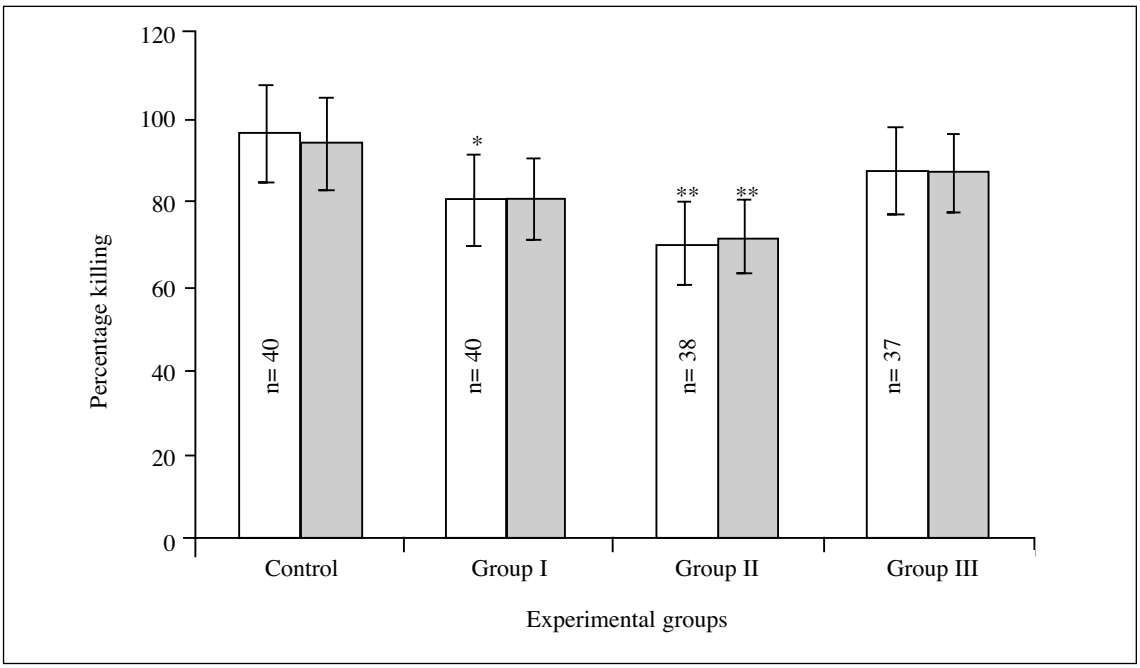

Figure 2. Bacterial killing exerted by PMNL (□) and MØ (▩) normal controls and in OBJ patients. The reaction mixture consisted of 1.6 mL lymphocytes (1.5 x 10^7 cells), 0.5 mL bacterial suspension (*S. aureus* 3 x 10^7) and 0.2 mL of human pooled serum were incubated at 37°C for 20 minutes. The sediments obtained after centrifugation dissolved in Hank's balanced salt solution and aliquots were spread in a nutrient agar plate and the colonies were counted. The values were expressed as percentage of control. Statistically significant difference are expressed as *p< 0.05 and ** p< 0.01 when compared to control.

Table 1. Determination of myeloperoxidase activity by o-toluidine and NBT staining

	% of cells stained by	
	O-toluidine	**NBT**
Control	89.7 ± 10.1	83.1 ± 9.1
Group I-Gall stone	60.5 ± 7.1*	73.9 ± 8.1#
Group II-Cancer in the head of pancreas	75.6 ± 9.1*	50.6 ± 6.1**
Group III-Bile duct stricture	83.6 ± 9.1#	65.6 ± 7.3*

Values are expressed as mean ± SD for 35 number of subjects in each group. Statistically significant variations are expressed as # p< 0.05, *p< 0.01 and **p< 0.001 when compared to control subjects.

A significant increase in 3H thymidine incorporation was seen in the lymphocytes isolated from obstructive jaundice patients when compared to that of normal subject and the change was highly significant after the addition of the mitogen conconavalin-A (Table 2).

DISCUSSION

Alteration of mucosal and systemic immune response may play an important role in the pathogenesis of hepatobiliary diseases. The aim of the study was to evaluate natural immune responses in patients affected by biliary obstruction and jaundice. Pha-

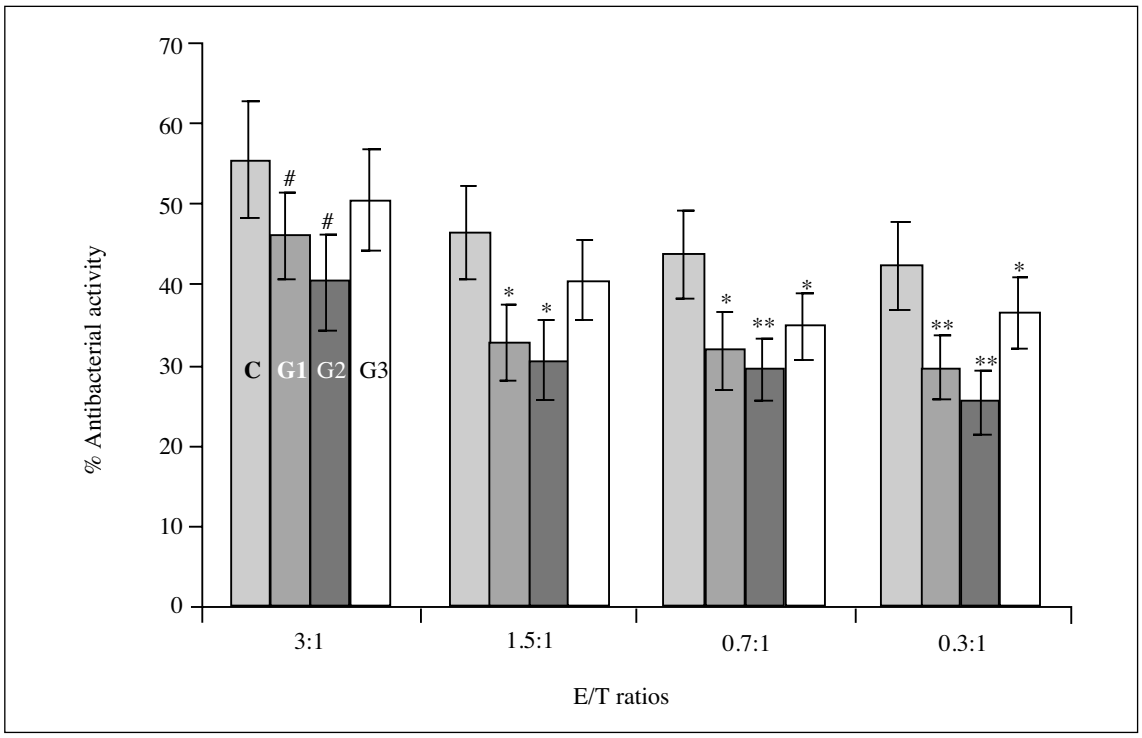

Figure 3. Lymphocytes mediated antibacterial activity in normal controls and OBJ patients. 0.2 mL of *Salmonella typhi* (1 x 10^4 cells, target cells) were mixed with PMNL (effector cells) at different effector/target ratios and incubated at 37°C for 2 hours. Aliquots of these mixture were then plated on dishes containing agar tryptose. After overnight incubation, colony forming units were counted. The results were expressed as percentage of normal. Values are expressed as mean ± SD for 35 individuals in each group. Statistically significant difference are expressed as $^{\#}p< 0.05$, * p< 0.01 and **p< 0.001 when compared to control subjects.

Table 2. Mitogenic stimulation index of lymphocytes isolated from control subjects and OBJ patients

Mitogenic stimulation index (DPM/2 x 10^5 cells)	Control	Group I	Group II	Group III
Blank	349.0 ± 46.1	340.8 ± 40.1	300.0 ± 43.1	326.0 ± 41.6
+ Con A	8900 ± 950	9421 ± 1041*	10251 ± 3051*	9000 ± 1000^{NS}
+ LPS	5650 ± 700	5981 ± 650*	4996 ± 521*	5551 ± 650^{NS}

Values are expressed as mean ± SD for 35 number of subjects in each group. Statistically significant variations are expressed as *p < 0.01 and NS: Not significant when compared to control subjects.

gocytosis of *C. albicans* exhibited by PMNL and MØ is a standardized model for the evaluation of natural immune responses for various diseases[22,23]. The low level of phagocytosis exhibited by both PMNL and MØ shows the functional impairment of lymphocytes in obstructive jaundice. Phagocytosis is an important first line immune defense process in which antigens are processed and presented to T-lymphocytes for further immune response.

The functions of lymphocytes are reported to be altered in various diseases[24,25]. Phagocytosis is directly related with oxidation stress and it has been reported that the

degree of phagocytosis decreases when there is increased free radical mediated oxidation stress[26]. So, the oxidation stress may be one of the reasons for the functional deficiency of lymphocytes in engulfing the pathogens. Phagocytosis has been associated with oxidative stress markers such as elevated levels of conjugated dienes, lipid hydroperoxides and lipid peroxides.

During phagocytosis respiratory burst in human monocytes is associated with increased uptake of glutathione[10]. Many hepatobiliary diseases have been treated successfully with the co-administration of anti-oxidants. For example, the protective effect of L-arginine against stress induced gastric mucosal lesions in rats and its relation to nitric oxide mediated inhibition of neutrophil infiltration have been reported[27]. Dietary carotenoids have positive role on the improvement of human immune functions[28]. The above mentioned reports support the findings of this investigation and it can be suggested that oxidation stress may be responsible for the impaired functions of phagocytic cells.

The level of percentage killing of bacteria inside the phagocytes was considerably low, indicating defects in either oxygen dependent or oxygen independent path way of killing the microorganisms. The activity of myeloperoxidase is an important factor in the microbial killing process inside phagocytes.

Myeloperoxidase is a oxidative enzyme expressed in polymorphonuclear leukocytes. It is involved in the bacterial killing action and is also able to mediate inflammatory tissue destruction in various diseases like brucellosis, vasculitis and peridontel diseases[29,30].

Myeloperoxidase activity has been reported to be decreased during oxidation stress and the observed decrease in bactericidal activity of both PMNL and MØ might be due to deficient myeloperoxidase activity to kill the pathogens by an oxygen dependent mechanisms inside the phagocytes[31].

Involvement of macrophages, monocytes and lymphocytes in the human immune defense mechanism is well established. Lymphocytes actively proliferate when encounters with antigenic stimuli. So, the level of proliferation of lymphocytes is a measure of immunity in health and diseases[32,33]. The results suggest that when lymphocytes are challenged with conconavalin-A in vitro, stimulation and proliferation exhibited by lymphocytes from obstructive jaundice patients were significantly high when compared to that of normal subjects. This shows that in obstructive jaundice subjects there is a increased activation and stimulation of lymphocytes which are required for both humoral and cell mediated immunity to fight against the pathogens.

It could be concluded that the functional impairment of lymphocytes may be responsible fort the immunity alterations and postoperative complications in obstructive jaundice patients.

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