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RAT PERIPHERAL BLOOD NEUTROPHIL LEUKOCYTES IN CHRONIC EXPERIMENTAL ALCOHOLISM. A MORPHOLOGIC AND FUNCTIONAL ANALYSIS

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In view of the presumed increased susceptibility of chronic alcoholics to infectious diseases, the influence of alcohol in vivo on some rat neutrophil cytochemical properties, such as chemotaxis, phagocytosis and ultrastructural morphology was investigated. Our observations suggested that chronic ingestion of large quantities of alcohol suppresses alkaline phosphatase, naphthol ASD chloracetate esterase, beta-glucuronidase and lysosyme enzymes in the peripheral blood neutrophils and at the same time enhances acid phosphatase but did not affect myeloperoxidase enzymes. In experimental rats the cytochemical scores of bactericidal cationic proteins and glycogen were reduced in circulating neutrophils, but the phospholipid score was increased. Phagocytic function, such as endocytosis, as well as reduction of nitroblue tetrazolium dye were not impaired by exposure to alcohol, but alcohol intoxication caused poor chemotactic activity (both spontaneous migration and chemotactic response). Ultrastructural changes were observed in mitochondria, such as clumping, elongation, swelling and disruption of cristae, as well as changes in the topographic distribution of cristae, as well as changes in the topographic distribution of granules in the cytoplasm. For example, cytoplasmic areas with numerous granules were registered together with other areas with a smaller number or without any granules. There was general distortion and widening of the cisternae of the endoplasmic reticulum. The neutrophils of ethanol – treated rats were atrophic.

These results suggest that a combination of morphological and dysfunctional changes in neutrophils may be responsible for some of the immunological alterations observed in chronic alcoholics.

Key words: Alcohol, Neutrophyl leukocytes, Function, Morphology, Rat

INTRODUCTION

Consumption of large amounts of ethanol has been associated with a number of health problems, including an increased incidence of infectious disease (reviewed by MacGregor, 1986; reviewed by Ballard, 1989; Mendenhall et al., 1990; Steven et al., 1990; Jerrells et al., 1992), liver disease (Lieber, 1990), and cancer (reviewed by Breeden, 1984). Study findings obtained with several diverse approaches have established that ethanol abuse is associated with a loss of immunocopetence. This loss may be at least partially responsible for the increased incidence of infectious disease and tumors. Alterations in the immune system involve predominantly abnormalities of lymphocytes (Glassman et al., 1985) and monocyte – macrophages (Bagasra et al., 1988) . Although numerous authors have commented on the toxic effect of alcohol on polymorphonuclear neutrophil (PMN) function (MacFarland and Libre, 1963; Corberand et al., 1989; MacGregor, 1990) their findings are inconsistent and to some extent contradictory. In comparison to other cell types related to immunity, relatively little is known about the effect of alcohol on leukocytes. Because alcohol predisposes to infection and increases its severity the major focus of research has been the neutrophil, the primary defense cell against bacterial invasion.

However, whether the altered immune response is due to the effect of alcohol *per se*, or to the other frequently associated complications of alcoholism (I. E. nutritional deficiencies, liver dysfunction, lower socioeconomic lifestyle, etc.) has not been determined. There are only a few documented studies in man or in experimental animal models where alterations in the immune system were determined to be directly correlated with chronic alcoholism only, because the other associated factors mentioned above were well controlled.

In patients it is very difficult to control the various confounding factors and directly correlate neutrophil alterations with ethanol consumption. Moreover, only a few of the studies in animal models adequately reflectet the conditions present in human chronic alcoholism such as duration of ethanol ingestion, route of administration, etc. (Astry et al., 1983; Marietta et al., 1988; Todorović et al., 1994).

In the present study we have examined neutrophil function and morphology at optic and electron microscopy levels, during the course of chronic ethanol consumption in a well-established model of experimental chronic alcoholism in the rat (Hartroft, 1971; Azzalis et. al., 1992) in which malnutrition, infectious and underlying hepatic dysfunctions were well controlled.

MATERIALS AND METHODS

Animals. Forty five adult male Wistar rats (Institute Colony Vinča), weighing about 240 g at the start of the experiment, were maintaind in single cages on a 12 hr light-dark cycle at 21 ± 1^0C. The animals were divided into three groups according to the diet offered, with 15 animals in each group: control *ad libitum* (C ad lib), control pair fed (C pair fed) and ethanol (E).

Alcohol administration. Control rats were fed a commercial stock cereal based pelleted diet (25% protein) as defined by Bieri et al. (1977). The ethanol

group was given free access to a hyperprotein cereal based pelleted diet (34% protein) and an aqueous solution of 24% sucrose (w/w)- 32% ethanol (v/v) as recommended by Hartroft (1971).

Blood sampling. Samples were taken before alcohol administration (start of the experiment) and at 8 and 16 weeks during alcohol intake for all tests excluding ultrastructural examination which was carried out only at the end of the experiment. Samples for determining blood alcohol level (BAL), white blood cell (WBC) number, chemotaxis, phagocytic ability and for making smears for cytochemical investigations were taken from the tail vein. For preparing buffy coat specimens for ultrastructural examination, blood was drawn after cardiac puncture.

B A L.

BAL was determined using the Sigma diagnostic alcohol procedure (No.322- UV, Sigma Chemical Co., St. Luis, MD). The mean blood ethanol level was determined for all ethanol-treated rats studied, based upon multiple BAL determinations (at 10 a. m. on days 1, 15, 30, 45, 60, 75, 90, 105, and 120) performed throughout the 120-day exposure period. *WBC counts, peripheral blood leukocyte differential counts*.

WBC counts were evaluated by a standard method. Peripheral blood leukocyte differentials were determined by counting 300 cells on May-Grünwald-Giemsa stained smears.

Cytochemical investigations of neutrophils

Enzyme and cationic bactericidal protein scores. Myeloperoxidase (MO), neutrophil alkaline phosphatase (NAIP) and naphthol ASD chloracetate esterase (NASDA) scores were obtained on smears by the techniques of Silviera and Hadler (1978). Merker (in Dobreva, 1984), and Higgy-Burns-Hayhoe (in Hayhoe and Quaglino, 1980), respectively. Kits for neutrophil acid phosphatase (NAcP) and beta-glucuronidase(β-GLU) staining were available from Sigma Chemical Co, St. Louis, Missouri (Sigma diagnostic R acid phosphatase. Procedure No 386; Sigma diagnostic TM lymphocyte enzyme, Procedure No 180). A PAP technique was used for identification of lysozyme (Dako PAP Kit, Code No. K 504). The cationic bactericidal protein score was determined by the method of Šubič (1974). The intensity of the staining for each enzyme and cationic bactericidal protein was graded semiquantitatively on the scale of 1 + to 4+ per 300 neutrophils, and total scores were calculated.

Neutrophil function tests. Chemotaxis assay (spontaneous migration and chemotactic response). The chemotactic activity of PMNs was studied under agarose by the method of Nelson et al. (1975).

Phagocytosis assay. The incubation procedure has been described before (Ribakova, Orlova, Lebengarc, 1975). The phagocytic activity was estimated according to Aleksejeva and Volkova (1966) on the basis of the following parameters: percentage of phagocytic cells, phagocytic index (Hamburger), phagocytic number (Rait) and absolute index of ingestion.

Reduction of nitroblue tetrazolium (NBT) dye. NBT reduction was determined using the Sigma research procedure (Sigma research kits and reagents, Kit No. 840-W). Heparinized blood samples were incubated with buffered NBT solution. Smears were prepared, stained and examind microscopically to determine the percentage of neutrophils exhibiting intracytoplasmic deposits of NBT-formazan.

Electron microscopy. Leucocytes were separated by the sedimentation method previously described (Anderson, 1965). The buffy coat with leukocytes was fixed with 1.5% glutaraldehyde in 0.5M cacodylate buffer at pH 7.2 for 1 h, washed with 7% sucrose in buffer and postfixed in 2% osmium tetroxide at 4^{0C} for 1 h. Following that the pellets were dehydrated in a graded series of ethanol and embedded in epoxy resin. Ultrathin sections cut from the embedded cell suspension (using an LKB ultratome II) were contrasted with lead citrate and uranyl acetate prior to viewing with an Opton 109 electron microscope.

Statistical analysis. All results, excluding those for neutrophil nuclear lobulation, were analyzed using two-way analysis of variance (ANOVA) and Tuckey's t-test for multiple comparisons.

RESULTS

The average daily amounts of the whole diets, as well as the energy consumed as food ethanol and separately from, protein, fat and carbohydrate intakes are shown in Figure 1. There was no significant difference between the ethanol-treated rats and controls with regard to daily energy intake and energy

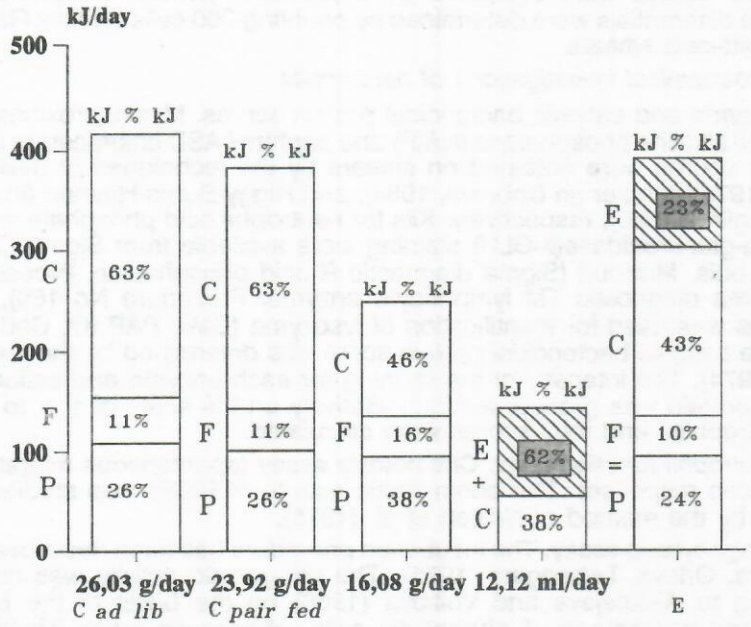

Figure 1. Average daily amounts of solid feed and fluid and percentage proportion of the different caloric ingredients consumed by the rats throughout the experiment. Foot notes for Figure 1.
The Figure shows the average daily amounts of basal diet (in g) consumed by the non-ethanol fed rats of group C ad lib (first column) and C pair fed (second column) and the average daily amounts of the hyperprotein diet (in g, third column) plus the amounts of sweetened ethanol mixture (in ml, fourth column) consumed by the ethanol-treated rats of group E throughout the 4- month experimental period. The fifth column chows the energy proportion of the nutrients in the final regimen (hyperprotein diet + ethanol-sucrose mixture) of group E. The percentage of energy derived from different nutrients are indicated inside the columns: C= carbohydrate, F=fat, P=protein and E=ethanol.

from protein, and fat. The average ethanol intake of group E was 3.09 g/day or 8.42 g/kg/day (data not shown) which was 23% of the daily energy intake.

At a given time animals of all groups had similar weights and body mass increased during the experiment (Figure 2).

The blood ethanol levels after the 8 and 16 week experimental periods were 108±6 mg% and 110±5 mg% respectively, and ranged from 105 mg% (data not shown).

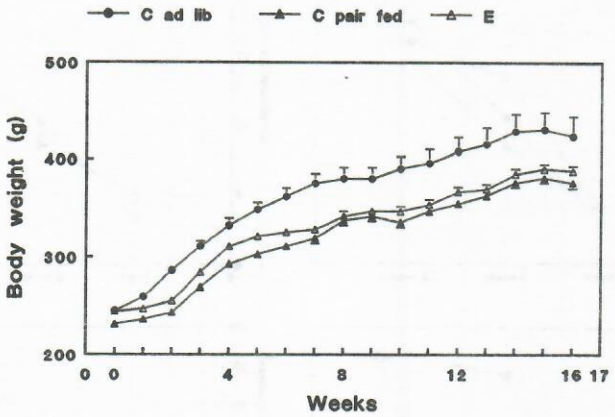

Figure 2. The body weight gain of the rats during the experiment.

The results for the absolute leukocyte count and relative number of different cell types are given in Figure 3. The differences in the mean leukocyte count were not statistically significant, but there were changes in the relative proportion of the various WBC types. Treatment of the rats with ethanol resulted in a significant increase in the number of PMNs in the peripheral blood after 8 and 16 weeks, and a tendency for a decrease in the number of lymphocytes and monocytes at the end of the experiment.

A summary of the quantitative analysis of cytochemical enzyme and cationic bactericidal protein reactions of circulating neutrophil leukocytes at different times during ethanol intake is shown in Table 1. There were significant differences between the results for controls and ethanol treated animals with respect to the following parameters studied: NAlP, NASDA, NacP, bGLU, lysozyme and cationic bactericidal protein score. The NAlp, β-GLU and lysozyme scores were lower in alcohol-treated animals that the same scores of control rats, and an interactive effect of ethanol and time (duration of experiment) was observed for all mentioned enzymes. In addition, an independent effect of time was observed for β-GLU scores. Cationic bactericidal protein content decreased

significantly in the alcoholic animals, and an interactive effect of alcohol and time was observed. NASDA and NAcP scores were reduced during the experiment, and an interactive effect of ethanol and time was observed. No differences were observed between alcoholic and control rats with respect to MO score, but an independent effect of time was observed (MO score was reduced in PMNs of all examined animals during ageing).

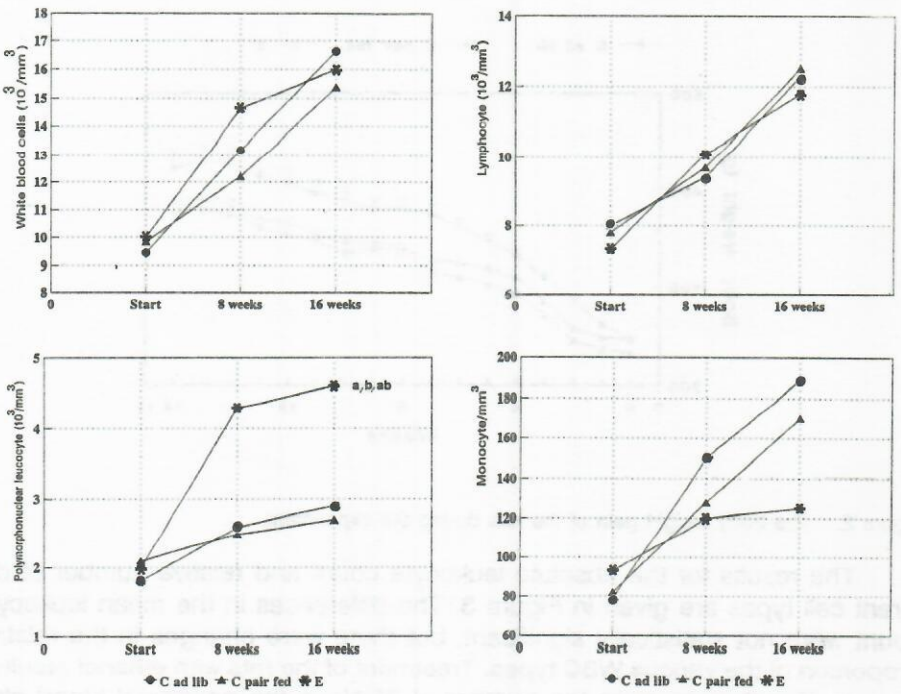

Figure 3. Effects of ethanol intake on the peripheral blood leukocyte differential cell count. Significantly different ($p<0.05$): a=ethanol effect; b=time effect; ab=interactive effect

As shown in Table 2, most of the neutrophil functions tested in this protocol were unaffected under the alcohol regime. The only function to be significantly affected was chemotactic activity (both spontaneous migration and chemotactic response).

The results of our ultrastructural investigation indicate that PMNs of alcoholic rats showed defective subcellular properties. Circulating polymorphonuclear leukocytes of control rats had a characteristic ultrastructural appearance (Figure 4).

Table 1. Cytochemical enzyme and cationic bactericidal protein reactions of rat circulating neutrophil leukocytes at different times during alcohol intake

	Time during alcohol intake		
	Start	8 weeks	10 weeks
Myeloperoxidase (score)[b]			
C ad lib	244±3*	239±3	226±4
C pair fed	240±3	233±4	230±4
E	249±3	234±4	230±4
Alkaline phosphatese (score)[a, ab]			
C ad lib	229±11	223±6	230±9
C pair fed	230±12	233±12	226±13
E	231±12	226±6	175±8
Naphthol ASD chloracetate esterase (score)[b, ab]			
C ad lib	275±7	275±7	269±8
C pair fed	270±8	272±8	263±9
E	275±7	275±6	235±8
Acid phosphatase (score)[b, ab]			
C ad lib	173±5	172±4	166±4
C pair fed	171±5	176±5	171±6
E	171±5	170±4	199±1
β-glucuronidase (score)[a, b, ab]			
C ad lib	126±8	125±8	125±5
C pair fed	124±5	125±3	125±5
E	127±9	123±9	79±9
Lysozyme (score)[a, ab]			
C ad lib	275±8	272±8	269±8
C pair fed	270±9	279±6	272±7
E	273±7	275±6	240±8
Cationic bactericidal proteins (score)[a, ab]			
C ad lib	150±3	153±2	151±2
C pair fed	148±3	156±4	153±4
E	149±2	140±2	131±2

* $\bar{X} \pm$ SEM;
Significantly different ($p < 0.05$): a= ethanol effect; b= time effect; ab= interactive effect

A significant decrease of cell profile area and cytoplasm profile area was shown in ethanol treated rats. The results also showed that there was a significant effect of ethanol on the total number of cytoplasmic granules; in addition, there were changes in the topographic distribution of granules in the cytoplasm such as registration of cytoplasmic areas with numerous granules and areas with a smaller number or without any granules. Changes were observed in mitochondria such as clumping, elongation, swelling, and disruption of cristae. There was general distortion and widening of the cisternae of the endoplasmic reticulum (Figure 4). Some neutrophils of ethanol treated rats had autophagic vacuoles.

Table 2. Chemotactic activity, phagocytosis of E. coli and ability to reduce NTB in polymorphonuclear neutrophils of alcoholic and control rats

	Time during alcohol intake		
	Start	8 weeks	16 weeks
Chemotactic activity			
Spontaneous migration[a] (mm/3 hr)			
C ad lib	1.06±0.06	1.09±0.05	1.10±0.06
C pair fed	1.09±0.04	1.06±0.04	1.06±0.04
E	1.08±0.05	0.82±0.03	0.62±0.04
Chemotactic response[a]			
C ad lib	1.71±0.08	1.76±0.07	1.69±0.07
C pair fed	1.67±0.07	1.63±0.08	1.75±0.09
E	1.70±0.09	1.20±0.07	1.03±0.10
Phagocytic ability			
Percentage of phagocytic cells			
C ad lib	76.5±0.8	77.0±0.8	79.0±0.7
C pair fed	78.0±1.0	77.6±0.8	77.4±0.8
E	77.5±0.8	77.3±0.6	76.9±0.8
Phagocytic index (Hamburger)			
C ad lib	9.9±0.2	9.6±0.2	9.9±0.2
C pair fed	9.8±0.2	9.4±0.2	9.8±0.2
E	10.0±0.2	9.7±0.2	9.8±0.2
Phagocytic number (Rait)			
C ad lib	14.0±0.2	13.9±0.3	13.8±0.3
C pair fed	13.8±0.3	13.6±0.3	13.8±0.3
E	13.3±0.3	13.3±0.2	13.6±0.3
Absolute index of ingestion (x 10^9)			
C ad lib	18.2±2.6	23.2±2.0	27.0±3.0
C pair fed	17.6±2.5	23.9±2.2	28.0±3.5
E	19.9±1.6	42.4±5.0	47.0±6.2
NBT reduction (% of cells with positive NTB reaction)			
C ad lib	70.0±0.4	72.0±0.5	71.0±0.5
C pair fed	72.0±0.5	76.0±0.4	72.0±0.5
E	69.0±0.4	74.0±0.3	70.0±0.3

* $\bar{X} \pm$ SEM; Significantly different ($p<0.05$): a=ethanol effect

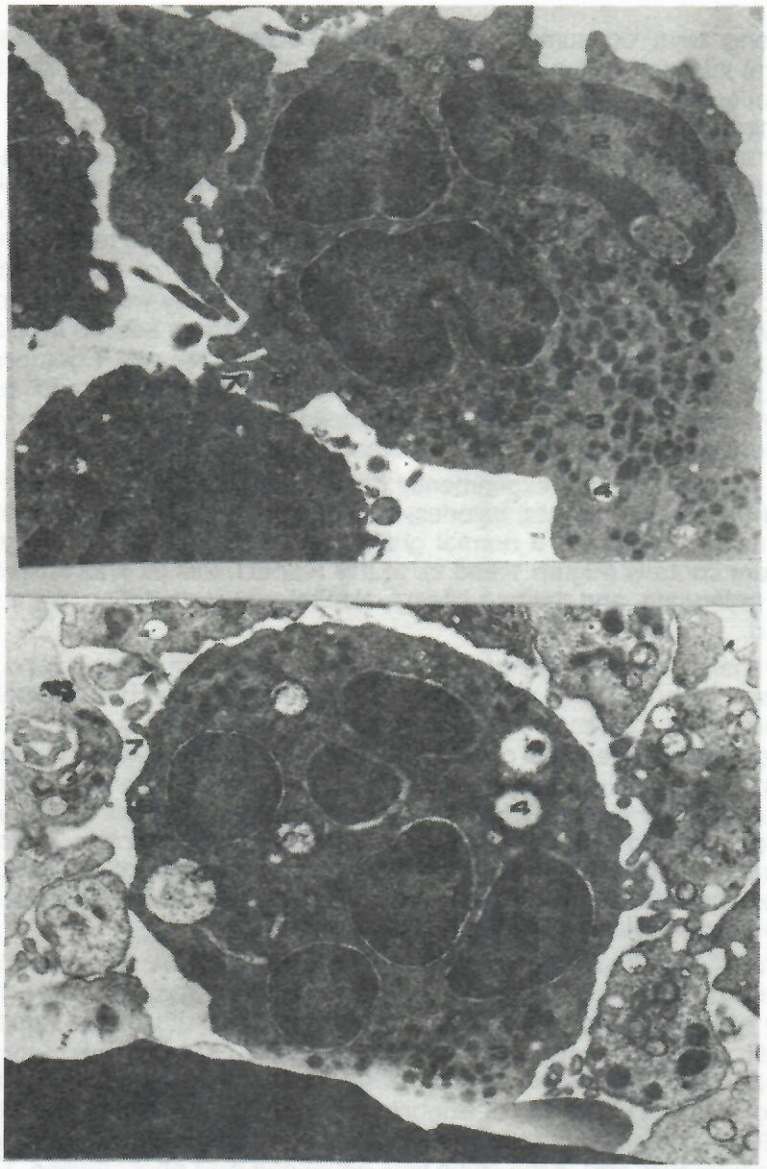

Figure 4. Neutrophil polymorphonuclear leukocytes of control (upper) and ethanol treated rats (lower).
1. heterochromatin of multilobulated nucleus; 2. euchromatin; 3. granule; 4. mitochondria; 5. endoplasmic reticulum; 6. pinocytic vesicles; 7. microvilli; 8. pseudopodia. Uranyl acetate, lead citrate, TEM, x 14000.

DISCUSSION

Long term consumption of ethanol causes increased susceptility to microbial infection which is possibly a result of ethanol-induced alterations in the functioning of immunocompetent cells after exposure to ethanol in the systemic circulation or in organs and a decrease in vitro functions of immune effector cells. However, it is difficult to gain a clear picture in humans, probably because of the interference of factors such as liver disease and/or malnutrition in the groups examined. These two conditions can independently or synergetically induce alterations in immune functions. In an attempt to avoid this kind of interference in the evaluation of the intrinsic effect of ethanol abuse on neutrophil morphology and function, in the present study we examined chronically alcoholic rats in good nutritional status and without serious liver damage. Since phagocytes (granulocytes and macrophages) are major components of the host defence mechanism against infections, quantitative and qualitative abnormalities of these cells in the alcoholic could play a major role in the pathogenesis of decreased host resistance to infection.

It is clear from our experimental design that rats given a chronic dose of alcohol (about 25% of total calories) do not consume inadequate amounts of other nutrients and had a normal growth rate when compared to pair fed or ad libitum controls (Figure 1 and 2). In this respect, this ethanol diet was nutritionally adequate, and the two effects, alcohol and malnutrition, were not confounded.

Elthanol had no effects on the total number of white blood cells in peripheral blood. The observations of an increase in number of PMNs and a tendency for a decrease in the number of lymphocytes suggested that the peripheral blood differential cell count may exhibit subtle changes in the immune system accompanying exposure to ethanol manifested by a loss of circulating lymphocytes. Marietta et al., (1988) have reported that ethanol inhibition altered the relative proportion of lymphocytes and polymorphonuclear leukocytes in periphral blood and suggested that although no changes occurred in CFU-GM, perhaps the relative intensity to ethanol of the CFU-GM was related to the relative granulocytosis noted in the peripheral blood.

We determined neutrophil MO, NAIP, NAcP, NASDA, lysozym and β-GLU activities using the cytochemical semiquantitative scores. Although neutrophil activity of the MO enzyme was not affected by alcoholism, the same cells of alcoholic rats had a decreased NAIP, NASDA, β-GLU and lysozyme activity, while NAcP activity was increased. These observations suggested that chronic ingestion of large quantities of alcohol suppresses alkaline phosphatase, naphthol ASD chloracetate esterase, β-glucuronidase and lysozyme in the peripheral blood neutrophils and at the same time enhanced acid phosphatase. The effect of in vitro alcohol incubation on PMN lysosomal enzyme release in response to degranulation stimuli has not been evaluated enough nor have enzyme concentrations been measured in PMN exposed to alcohol in vivo. MacGregor and colleagues (1988) have shown that neither resting cell concentration of granule contents nor the degree of their spontaneous release were affected by exposing

PMNs to alcohol in vivo. Corberand et al. (1989) examined neutrophil function in healthy volunteers who had taken a single large dose of whisky. Before and at different times after ingestion, several PMN properties were simultaneously tested including myeloperoxidase and neutrophil alkaline phosphatase scores. These properties were unaffected after alcohol ingestion. To our knowledge, this is the first report of measurement of neutrophil NAcP, NASDA, lysozyme and β-GLU activities in alcoholism. Aldehyde dehydrogenase, glucose-6-phosphate dehydrogenase and pyruvate kinase activities were determined in erythrocytes of various ages in alcoholic patients by Mezey and Rhodes (1988). The findings suggested that chronic alcohol ingestion suppressed aldehyde dehydrogenase, while it enhances glucose-6-phosphate dehydrogenase and pyruvate kinase. Mezey and Rhodes hypothesized that the decrease in enzyme activity in alcoholics indicated decreased synthesis of the enzymes and/or enzyme inactivation. This conclusion may be the basis for an explanation of our observations of depressed NAlP, NASDA, lysozyme and β-GLU enzyme activities after chronic alcohol ingestion. There was a paradoxical increase in neutrophil acid phosphatase enzyme activity and the mechanism for this increase is unexplaind. Also, there were independent time effects with respect to the scores of MO and β-GLU and the interactive effect of alcohol and time with respect to scores of NAlP, NASDA, β-GLU and lysozyme. The studies of Perskin and Cronstein (1992) suggest that an age-related decrease in plasmic membrane viscosity is associated with a decrease in 0_2 production and adherence of neutrophils to components of the extracellular matrix. Borzi et al., (1992) suggest that intracellular nucleotides (ATP, UDP, CTP and UDP-glucose) accumulate in aged subjects because of altered biochemical pathways.

Cationic bactericidal protein content significantly decreased in the alcoholic animals and an interactive effect of alcohol and time was observed. No study of neutrophil cationic bactericidal protein has been undertaken in chronic ethanol intake, and we concluded that this change may be correlated with direct cellular toxicity.

As shown above, most of the neutrophil functions tested in this protocol (phagocytic ability and NBT reduction) were unaffected under the alcohol regime. The only function to be significantly affected was chemotactic activity (both spontaneous migration and chemotactic response). Phagocytic functions such as endocytosis and intracellular killing are not impaired by either in vitro (Hallengren and Forsgren, 1978) or in vivo (MacGregor et al., 1978; Astry et al., 1983; Corberand et al., 1989) exposure to relevant concentrations of alcohol. However, chronic alcoholics admitted for withdrawal treatment have a smaller chemotactic response than do normal controls (MacGregor et al., 1978). Moreover, half the group of alcoholics drinking daily in a controled environment had decreased chemotaxis (Gluckman et al., 1977). Most studies indicated that PMNs from the patients remained capable of responding to normal chemotactic factors, but a recent article reports a cellular chemotactic defect in 60% of patients with cirrhosis; the investigators theorized that the PMN defect is related to chronic in vivo activation of PMNs by C_5a generated in turn by circulating immune complexes (Rajkovic et al., 1984).

Decrease in the cell profile area and a large number of highly segmented neutrophils were present in the peripheral blood of the ethanol treated rats. These results suggested that such neutrophils were atrophic. Changes were observed in the mitochondria such as clumping, elongation, swelling, and disruption of cristae. There was general distortion and widening of the cisternae of the endoplasmic reticulum. Mitochondrial function appears to be an early target for ethanol toxicity. It is well established that the oxidation of ethanol in the liver and the heart may lead to mitochondrial dysfunction (Quintanilla and Tampier, 1989; Cunningham et al., 1989).

The results of our study indicated that the described combination of morphological and dysfunctional changes may be correlated with both direct cellular toxicity and adaptation to ethanol.

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NEUTROFILNI LEUKOCITI PERIFERNE KRVI PACOVA U HRONIČNOM EKSPERIMENTALNOM ALKOHOLIZMU

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SADRŽAJ

Morfološka i funkcionalna analiza korišćena je za procenu citohemijskih, subcelularnih i funkcionalnih poremećaja polimorfonuklearnih leukocita periferne krvi u 15 pacova hronično tretiranih etanolom i 5 kontrolnih životinja koje su bile na standardnoj adekvatnoj izoenergetskoj ishrani. Eksperiment je trajao 4 meseca. Hronični unos alkohola suprimirao je aktivnost enzima alkalne fosfataze, naftol ASD hloracetat esteraze, beta-glukuronidaze i lizozima, dok je u isto vreme aktivnost kisele fosfataze bila znatno povećana, a mijeloperoksidaze nepromenjena (procenjeno na osnovu semikvantitativne citohemijske evaluacije). U eksperimentalnih životinja bili su redukovani citohemijski skorovi sadržaja baktericidnih katjonskih proteina i glikogena, dok je isti skor za fosfolipide bio povećan. Fagocitna funkcija, procenjena preko testa endocitoze, kao i redukcija nitroblue tetrazolium-a bili su neoštećeni, a hemotaksna aktivnost (spontana migracija i hemotaksični odgovor) je bila suprimirana u eksperimentalnih životinja. Ultrastrukturna ispitivanja su pokazala proširenje cisterni endoplazmatskog retikuluma; u nabubrelim i uvećanim mitohondrijama primećeno je ekstremno prosvetljavanje matriksa, liza membrana i disrupcija kristi;postojao je manji broj granula diskontinuirano raspoređenih u citoplazmi. Ovim nalazima može se podržati koncept da kombinacija različitih morfoloških i funkcionalnih poremećaja u neutrofilima može biti delom odgovorna za imunološke poremećaje primećene u hroničnom alkoholizmu.