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Macrophage function in chronic experimental alcoholism

I. MODULATION OF SURFACE RECEPTORS AND PHAGOCYTOSIS

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SUMMARY

In the present study, we have assessed peritoneal, alveolar and splenic macrophages for expression of Fc and C3b surface receptors and their ability to function in immunophagocytosis. We have also measured their oxidative burst response by the nitroblue tetrazolium dye (NBT) reduction method. Our studies revealed that macrophages harvested from chronic alcoholic rats expressed surface C3b and Fc receptors, with significantly higher surface density than macrophages of litter-mate controls (matched for sex and nutritional calories). However, the ability of macrophages from alcoholic rats to phagocytize through C3b and Fc receptors was significantly impaired. In addition, the ability of peritoneal macrophages from alcoholic animals to ingest non-opsonized *Candida albicans* and to reduce NBT dye was markedly compromised. Abnormalities of macrophage function may, at least in part, account for an increased susceptibility of alcoholic patients to infection

INTRODUCTION

The literature is replete with reports of a marked increase in the frequency of serious infections in chronic alcoholics (Adams & Jordan, 1984; MacGregor, 1986). However, there is little direct experimental evidence concerning the cellular basis for alcohol-associated infections. In a recent publication we have shown decreased function and numbers of T-cell subpopulations in an animal model of chronic alcoholism (Bagasra *et al.*, 1987). We report here our findings of the function of macrophages in this experimental model.

Immunophagocytic function of macrophages is important in host defence against various pathogenic micro-organisms. Immunophagocytosis involves the presence of high-affinity surface receptors for the Fc portion of IgG (FcR) and for C3b (C3bR). Uptake and internalization of Fc- and C3b-bound particles involves formation of plasma membrane-derived vesicles that enclose the particles complexed with the surface receptors. Microbicidal mechanisms then involve primarily two major cellular events: enzyme release into these vesicles and initiation of the oxidative burst. The oxidative burst metabolic pathways have a highly reactive microbicidal effect. Activation of these oxidative burst pathways can be assessed by the NBT dye reduction test. NBT is a yellow-coloured water-soluble electron acceptor dye that, on reduction, is converted to blue formazan, an insoluble blue compound.

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We assessed here functional aspects of a macrophage population harvested from chronic alcoholic animals, and compared them to those of litter-mate controls. We studied: (i) surface densities of FcR and C3bR (as determined by FcR- and C3b-mediated binding activity); (ii) phagocytic capacities of macrophages through FcR and C3bR; and (iii) NBT reduction by products of the oxidative metabolism in macrophages after stimulation with *C. albicans*.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA), initially weighing 150–160 g, were fed a totally liquid diet in which ethanol provided 36% of total calories, protein 16%, fat 35% and carbohydrate the remainder. Pair-fed rats received the same diet, except that carbohydrate isocalorically replaced ethanol. Rats used in this study were maintained on this diet for the duration of the study (12 weeks). Ethanol consumption averaged $14{\cdot}0 \pm 0{\cdot}6$ (SD) g per kg of body weight per day. Details of biochemical and histopathological changes during liquid diet have been described previously by others (DeCarli & Lieber, 1967; Thayer & Rubin, 1979; Tarachi & Rubin, 1985).

Macrophage cell collection

Resident peritoneal macrophages were collected after 10 ml injection of cold PBS into the peritoneal cavity and careful massage of the abdomen for 5 min (Edelson & Cohen, 1976). Pulmonary alveolar macrophages were harvested by the technique described by Fisher *et al.* (1978). Splenic macrophages

were harvested by carefully teasing the spleen into a single cell suspension.

All cell suspensions were made in RPMI-1640 medium containing 10% fetal calf serum and 50 μg/ml Gentamicin (complete medium). Cell clumps and debris were removed by sedimentation at 1 *g*; contaminating erythrocytes were removed by hypotonic lysis. All cells were subsequently washed twice by centrifugation and resuspended in complete medium.

Preparation of monolayers of macrophages

The cells were adjusted to a concentration yielding 10^5 macrophages per 12 mm glass coverslip. The coverslips were placed within 16 mm wells of 24-well sterile plastic tissue culture plates (Costar, Cambridge, MA). After incubation for 2 hr at 37° in humidified air containing 5% CO_2, individual coverslips were removed with forceps, immersed in warm RPMI (37°), and agitated vigorously to remove non-adherent cells. An additional washing was performed with forced flushes of warm RPMI to remove any residual non-adherent cells.

The adherent cells were 96–98% macrophages, as determined by morphology, ingestion of 0·81 μm latex beads (Difco Laboratories, Detroit, MI) and non-specific esterase (NSE) staining in normal control monolayers (Koski, Poplack & Blaese, 1976). The percentage of cells showing morphology and staining properties did not change with the alcoholic diet.

Assays for macrophage FcR and *C3bR densities*

The presence of FcR was determined by using IgG-coated sheep red blood cells (SRBC). Washed SRBC (Hazleton, PA) were diluted to a 5% suspension in phosphate-buffered saline (PBS). Two millilitres of this preparation were added to 50 μl of a subagglutinating dilution of the IgG-anti-SRBC serum. This suspension was mixed and incubated for 30 min at 37°, after which time the cells were washed three times with PBS and then resuspended in 5 ml of veronal-buffered saline (VBS). To 24-well plates containing coverslips with monlayers of macrophages, 250 μl of this preparation were added and incubated for 1 hr at 37° in 5% CO_2 and 95% air. At the end of the incubation period the unattached sheep erythrocytes were removed by gently washing the wells three times with PBS. Then the cells were fixed with 2% gluteraldehyde for 10 min and stained with counterstain. For the counterstain, either 0·5% methyl green or Giemsa–Wright stain (Diff-Quick, Harleco, Gibbstown, NJ) was used. Macrophages were enumerated for the percentage of Fc rosette-forming cells. At least 200 cells were counted in each coverslip, and rosettes were defined as macrophages attached to at least three RBCs.

The presence of complement receptors (C3bR) on macrophages was determined by using sheep erythrocytes coated with IgM and complement. The sheep erythrocytes were opsonized, first with subagglutinating dilution of rabbit anti-SRBC IgM by incubating 2 ml of washed SRBC (5% suspension) with 50 μl of (predetermined dilution) anti-SRBC IgM serum for 30 min at 37°. The SRBC were then washed with PBS three times and resuspended in 1 ml of VBS containing 0·1 ml of fresh normal rat serum and 0·1 ml of Suramin solution (2 mg/ml; Suramin, Bayer 205, Bayer Pharmaceutical Inc., FRG). Suramin inhibits the conversion of C3b and C3bi (Bianco, 1976). The suspension was mixed well and incubated for an additional 10 min at 37°, washed three times with PBS and resuspended in 5 ml of veronal-gelatin buffer (VGB). The percentage of macrophages with C3bR was evaluated by counting the proportion of cells that adhered to three or more SRBCs. Controls consisted of sheep erythrocytes coated with anti-SRBC (IgM) alone. These control values did not exceed 5% of the C3b IbG-mediated rosette values.

In order to determine the density of FcR on the surface of macrophages, SRBC were coated with progressively decreasing concentrations of anti-SRBC IgG (Serio, Gandoer & Walker, 1979). The concentration of IgG was chosen in preliminary experiments: we used the lowest concentration of IgG that could still elicit rosette formation in about 5% of peritoneal macrophages of normal animals. Similarly, in the assays for density of C3bR on macrophages, decreasing concentrations of anti-SRBC IgM were used, but the source of complement (fresh serum) was maintained constant. Even though in this method the values are expressed as a percentage of cells that will bind SRBC, the assay actually measures receptor density/affinity on the cell surface among different macrophage populations, since cells with a high density of surface receptors will bind SRBC to small amounts of Fc or C3b. In these assays, an increase in affinity of the surface receptors for their ligands cannot be distinguished from an actual increase in the surface receptor density.

Fc- and C3b-mediated ingestion assays

Macrophage immunophagocytic function was assessed by using either Fc or C3b receptor-mediated ingestion activity, as originally described by Bianco, Griffin & Silverstein (1975) with minor modifications (Tabor, Azedegan & LeFrock, 1985). Briefly, washed SRBCs were opsonized with either a subagglutinating concentration of IgG (FcR-mediated phagocytosis) or with 19S IgM rabbit anti-SRBC and fresh serum as a source of complement (C3bR-mediated phagocytosis). Ingestion controls, consisting of SRBC alone, were used to identify non-specific ingestion. Cells were incubated at 37° for 2 hr. Slides were examined microscopically under oil immersion, scoring cells with two or more ingested erythrocytes as positive. Between 500 and 1000 cells were counted per slide. The results were reported as the percentage of macrophages ingesting SRBC coated with IgG or SRBC coated with IgM and complement (Fc- or C3b-mediated phagocytosis, respectively) minus the background, which did not exceed 5% of the SRBC. Negligible ingestion of SRBC alone was observed throughout the assays, indicating that artifactual and non-specific erythrocyte phagocytosis was minimal.

Phagocytosis of C. albicans

In order to assess the percentage of phagocytic cells in adherent cell populations from various sources, 50 μl of a *C. albicans* suspension containing $1{\cdot}0 \times 10^7$ organisms in PBS were added to each 24-well plate and incubated for 8 hr at 37°. Subsequently, dishes were washed five times with PBS and the cells fixed with 2% gluteraldehyde for 10 min, and stained with NBT or Giemsa–Wright stain.

Reduction of nitroblue tetrazolium dye by macrophages

Nitroblue tetrazolium (NBT), a yellow water-insoluble dye, forms blue insoluble granules that are focused at the site of their production in phagocytes undergoing the oxidative burst (Baehner, Boxer & Davis, 1976). This activity has been shown to correlate with enhanced microbicidal function (Babior, 1978).

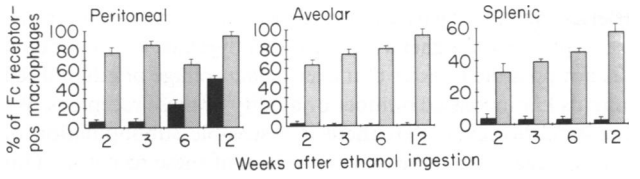

Fig. 1. Modulation of the expression of Fc receptors in various macrophage populations (peritoneal, alveolar, splenic). Percentage of macrophages forming rosettes with SRBC sensitized by limiting amounts of anti-SRBC IgG (EA) as described in the Materials and Methods. Bars represent the mean percentage +1 SD. Dark bars, litter-mate controls; light bars, ethanol-fed animals.

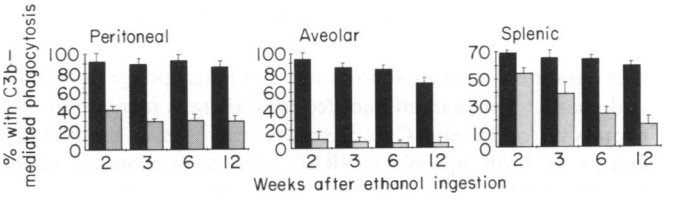

Figure 2. Phagocytosis of SRBC opsonized by IgM and C3b (EAC) by various macrophage populations. Bars represent the mean percentage +1 SD of macrophages ingesting two or more erythrocytes after 2-hr incubations as described in the Materials and Methods. Dark lines, litter-mate controls; light bars, ethanol-fed animals.

Analysis of the NBT reductive capacity was done on macrophages using the procedure described by Hedley & Currie (1978): macrophage monolayers on 12-mm glass coverslips contained within 24-well tissue culture flasks were incubated with *C. albicans* suspension plus 1 ml of NBT (1 mg/ml) in RPMI medium for 8 hr at 37° in an incubator. During the first 30 min of incubation, plates were gently rocked at 3 cycles/second followed by a 7½-hr incubation period without rocking. Subsequently, the macrophage-containing coverslips were removed from the wells. Excess unattached *C. albicans* were removed by vigorous rocking and coverslips were mounted on glass slides. Using oil immersion, microscopic analysis was performed by counting the distinct brown granules within macrophage vacuoles. Cells containing *C. albicans* and ≥ thre granules were scored as positive, and results were reported as the percentage of phagocytizing macrophages that were NBT positive. Between 500 and 1000 cells were counted per slide.

Statistical analysis

The results from each experiment were obtained by using three rats per experimental variable. Slides assayed for the phagocytosis, NBT, surface receptor density or phagocytosis were determined in triplicate for each individual animal. All paired data results were analysed by Student's *t*-test.

For calculations of correlations between *C. albicans* phagocytosis, SRBC phagocytosis and NBT activity of macrophages, the Pearson coefficient of correlation was used.

RESULTS

Macrophage Fc and C3b surface receptors

The macrophages from ethanol-fed animals and their litter-mate controls (matched for sex and nutritional factors) were assessed at Weeks 2, 3, 6 and 12 after the initiation of a liquid diet containing ethanol. Ethanol-fed animals and their litter-mate control experiments were run at the same time in identical conditions.

Assay values of expression of surface FcR density for peritoneal, alveolar and splenic macrophages from alcoholic animals were several-fold higher than their litter-mate controls (Fig. 1). Resident peritoneal macrophages of animals with normal diets increase FcR density as the animals get older (Fig. 1), but alveolar and splenic macrophages express relatively the same FcR density throughout the entire course of the study.

Surface receptors for C3b also exhibited an increase in their density during the course of chronic alcoholism, which paralleled the increase in FcR density (data not shown).

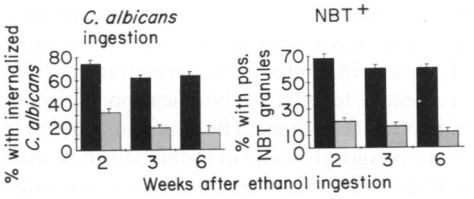

Figure 3. Phagocytosis of *C. albicans* and ability to reduce NBT in various populations of macrophages. Macrophages from alcoholic (light bars) and litter-mate controls (dark bars) were examined for their ability to show a reduction of NBT dye after ingestion of *C. albicans*, as described in the Materials and Methods. Bars represent the mean percentages +1 SD.

Fc- and C3b-mediated ingestion

Normal macrophages will efficiently ingest red blood cells opsonized with IgM plus complement through interaction with C3bR. As shown in Fig. 2, the peritoneal, alveolar and splenic macrophages harvested from ethanol-fed rats at 2, 3, 6 and 12 weeks after initiation of ethanol diet exhibited significant reductions in their ability to phagocytize opsonized SRBCs via C3b receptors. For example, less than 50% of peritoneal macrophages exhibited phagocytic ability compared with a litter-mate control value of approximately 90%. Phagocytic ability of these macrophages decreased significantly ($P<0{\cdot}05$) during the course of chronic alcoholism and at Week 12 only 30% of macrophages showed phagocytic capacity. Splenic macrophages from alcoholic animals exhibited a reduction in their phagocytic capacity similar to peritoneal macrophages. The most significant reduction in phagocytic ability was found in alveolar macrophages where less than 10% of alveolar macrophages showed phagocytosis.

Modulations in ingestion capacities of alcoholic macrophages through the Fc receptors paralleled the results observed for C3b-mediated ingestion (data not shown).

Capacity of macrophages to reduce NBT dye

Peritoneal macrophages from alcoholic animals were examined for their ability to reduce NBT dye and compared to those of their little-mate controls. It was observed that peritoneal macrophages harvested from alcoholic animals markedly lost their ability to reduce NBT dye compared with parallel controls (Fig. 3). The decreased degree of NBT reduction correlated positively with the decrease in phagocytosis of both *C. albicans* ($r_s=0{\cdot}96$, $P<0{\cdot}05$) and opsonized SRBC ($r_s=0{\cdot}91$, $P<0{\cdot}05$).

DISCUSSION

Our data show marked abnormalities of macrophage functions and characteristics in ethanol-fed rats: there is increase in the expression of Fc and C3b receptors, decrease in the phagocytosis of both opsonized SRBC and non-opsonized yeast particles, and a decrease in NBT reduction.

Numerous studies have been reported on the effects of ethanol on monocyte and macrophage phagocytic function in humans and in experimental animals. These studies generally show a decrease in phagocytosis: Liu (1979) demonstrated decreased clearance of radiolabelled aggregated human serum albumin from the circulation of alcoholic patients without overt clinical evidence of hepatic cirrhosis or infection. This was interpreted as an indication of depressed phagocytic function of the fixed macrophage system. The depressed phagocytic function returned to normal after one week of abstinence from ethanol. Louria (1963) injected bacteria in the peritoneal cavity of mice subjected to ethanol intoxication and demonstrated decreased bacterial clearance, a finding that could be interpreted as a defect in phagocytosis or in mobilization of macrophages. Gilhus & Matre (1982) showed that *in vitro* exposure to ethanol of normal human peripheral blood mononuclear cells decreases the phagocytosis of latex particles. Morland & Morland (1982) studied pair-fed rats for the effect of alcoholism on peritoneal macrophage function and found decreased phagocytosis of SRBC coated with IgG. Our data show similar findings, and expand these studies to show abnormalities of surface receptors and the production of free oxygen radicals (as assessed by NBT reduction).

Our studies and those of Morland & Morland (1982) have the advantages of using pair-fed animals. This model of alcoholism (DeCarli & Lieber, 1967) has several important advantages: (i) because ethanol is ingested orally, ethanol uptake and metabolism follow routes similar to those in human alcoholics; (ii) pair-fed litter-mates (matched for sex) are used as controls to reduce effects due to differences in caloric uptake and other nutritional aspects; (iii) animals fed such diets develop fatty livers, but do not develop hepatic cirrhosis; hepatic cirrhosis itself may cause immunologic alterations. Not all studies in the literature show decreased phagocytic function: Loose, Stege & DiLuzio (1975) subjected rats to acute oral administration of ethanol or Bourbon, but did not detect alteration in the intravascular clearance rate of ^{51}Cr-labelled SRBC. In that same study, however, Bourbon (but not ethanol) given for 21 days prior to the experiments induced a significant decrease in ^{51}Cr-labelled SRBC clearance rate. Two separate studies of phagocytosis of staphylocci by macrophages exposed *in vitro* to alcohol failed to show any alteration (Gee *et al.*, 1978; Brayton *et al.*, 1970). The clinical relevance of these and other studies using alcohol exposure *in vitro* are difficult to assess. The alterations that we are describing for alcohol exposure *in vivo* may be due to the action of ethanol metabolites generated by other types of cells not present in the *in vitro* system, or these alterations may occur only after more prolonged exposure to ethanol.

The expression of FcR and C3bR was markedly increased in ethanol-fed rats. Since we used limiting dilutions in order to determine the number of macrophages that showed Fc and C3b receptor activity, we did not detect the total number of cells bearing these receptors—the only macrophages that formed rosettes were those with a higher density of membrane receptors or receptors with higher avidity for their ligands. From our data it is not possible to infer that every macrophage of ethanol-fed animals has increased number or avidity of these receptors. We can state, however, that there is a sizeable subpopulation of macrophages with increased expression of these receptors. Our experiments do not address the mechanisms for this increased expression of receptors in animals fed with ethanol, therefore we can only make hypotheses about mechanisms. Chronic alcoholism has been reported to increase the order within lipid bilayers, conferring increased rigidity to the membrane (Darle, Ekholm & Edlund, 1970; Dawidowicz, 1985). This effect might induce an accumulation of receptors on the surface, or could alter the topographic distribution of these receptors or even change their spacial conformation, all of which could give the results we found. Another hypothesis is that ethanol could induce an increase in the expression of these receptors through its pharmacological actions on the regulators of cell activation, such as metabolites of arachidonic acid and cyclic AMP (Atkinson *et al.*, 1977; Salesse *et al.*, 1982, Horrobin, 1987; Rhodes, Salmon & Wood, 1984; Green & Claesson, 1986; Nagy *et al.*, 1982). Recently, Parent *et al.* (1987) have shown that ethanol exposure *in vitro* enhances the expression of class I MHC antigens (up to eight-fold) in a variety of cell lines and this increased expression of class I MHC antigens occurs with concomitant increase in RNA levels. Receptors for other types of ligands are modulated by ethanol in various tissues (reviewed by Bannister *et al.*, 1986).

Interestingly, even though the expression of FcR and C3b is increased in ethanol-fed animals, the phagocytic function facilitated by three receptors is decreased. Since there is deficiency in the phagocytosis of so many different types of targets, as shown in our experiments and in the literature, we favour the hypothesis that the initial receptor–ligand reactions are normal or even enhanced, but a subsequent step, common to all the various modalities of phagocytosis, is impaired by the use of ethanol.

Even after ingestion of *C. albicans* was completed by some of the macrophage of ethanol-fed animals, these cells were deficient in the ability to reduce NBT, a function usually interpreted as evidence for the formation of the superoxide anion (O_2^-) (Babior, 1978). At least in neutrophils (Lad *et al.*, 1985), and possibly in macrophages as well, superoxide production is decreased by prostaglandin E and low levels of cyclic AMP. Such alterations have been described in alcoholism (Atkinson *et al.*, 1977; Salease *et al.*, 1982; Horrobin, 1987).

Macrophages from different anatomical sites, as well as subpopulations within a specific site, vary considerably with respect to their cell surface characteristics and functions. In our studies, macrophages obtained by bronchoalveolar lavage showed a greater degree of difference between ethanol-fed and control animals than macrophages obtained by peritoneal lavage or from the spleen. This finding is consistent with a larger body of evidence in the literature, indicating a greater predisposition to pulmonary infections in the alcoholic than for infections in other organs (Adams & Jordan, 1984). None of the characteristics studied in macrophages of ethanol-fed animals showed a tendency to return to normal with the duration of the diet, and most got progressively worse. Some regional variation was noted, such as the alveolar macrophage phagocytic function mediated by C3b reached very low levels faster than did that of

splenic macrophages, but the significance of these findings are difficult to interpret.

Chronic ethanol consumption not only impairs important functions in the macrophages, but may also decrease the total number of available macrophages in some organs. In a previous paper, we demonstrated a decrease in the total and relative number of macrophages and monocytes (non-specific esterase-positive cells) in the spleen of rate on ethanol diet (Bagasra *et al.*, 1987).

Functional abnormalities in macrophage populations described here, if chronically applicable, may explain, in part, the increased incidence of infection in patients with alcoholism.

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