MATERIALS AND METHODS

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SUBJECTS Male habitual drinkers of age group 30-50 years with same socioeconomic background and with a history of 5-15 years of alcohol abuse were chosen for the study. Only those alcoholics were selected whose gamma glutamyl transpeptidase (GGT) values were higher than normal, thereby confirming hepatic damage, (Rosalki, 1975). There was no apparent clinical abnormalities in these subjects.

Normal subjects of the same age group, with same socioeconomic background and without any history of alcohol intake were also studied simultaneously as control.

After an overnight fasting for 12 hours,10 ml of blood samples were drawn from the subjects by vein puncture. A part of the blood was collected without anticoagulant to get the serum and a part in 3.8% sodium citrate for obtaining the cells.

ESTIMATION OF GAMMA GLUTAMYL TRANSPEPTIDASE (GGT) ACTIVITY IN SERUM Quantitative estimation of GGT activity in serum was carried out with the help of Enzopack reagent kit which is formulated on the Szasz method (Szasz, 1976) recommended by Scandinavian Society for Clinical Chemistry and Physiology.

The kit comprises of substrate (1 GGT) and buffer (2 GGT). First the working reagent was prepared by dissolving the contents of one bottle of reagent 1GGT (substrate) with 3 ml of reagent 2 GGT (buffer). 0.1 ml of test serum was mixed with 1 ml of working reagent and the first absorbance read exactly at 60 sec.and then the 2nd,3rd and 4th at an interval of 60 sec. with 405 nm wave length. The mean change in absorbance per minute (Δ A/min) was then determined for calculating test results.

Calculations

Serum GGT activity (IU/l) = Δ A/min x F where F = 1158 (calculated on the basis of molar extinction coefficient for paranitroaniline and sample to total volume ratio).

TEST FOR SERUM TOTAL PROTEIN Total protein in serum was determined by the Biuret method (Wootton and Freeman, 1982). For the test 0.2 ml of test serum(T) was added to 2.8ml of water and 5ml of biuret reagent. A blank(B) and a standard(S) was also prepared by adding 5ml of biuret reagent to 3ml of water and 3ml of known standard solution (5 g/l) respectively. The absorbance was read at 540 nm after 30 minutes, zeroing with the blank by using a colorimeter (Systronics, Model- Spectro Colorimeter 103, India).

Calculation

$$\text{Surum total protein (g/litre)} = \frac{\text{T-B (or test blank)}}{\text{S - B}} \times 5 \times 15$$

$$= \frac{\text{T - B}}{\text{S - B}} \times 75$$

TEST FOR SERUM ALBUMIN Serum albumin was determined by the dye binding method (Wootton and Freeman , 1982) . In this method 0.02 ml of test serum was added to 4ml of buffered indicator containing sodium citrate, citric acid and bromocresol green and mixed well. The absorbance was read immediately at 637 nm against a blank of buffered indicator. Albumin standards of known cocentration (60,40,30,20 10 g/l) were treated in the same way and the absorbance read immediately.

ELECTROPHORETIC ANALYSIS OF SERUM PROTEINS

For electrophoretic separation of different classes of protein in the serum, 20 μl of each serum sample were applied near middle portion of a strip of Whatman filter paper (Whatman filter paper no. 1) in vertical electrophoretic unit. Both the ends of the paper were dipped into barbital buffer of pH 8.6 and ionic strength of 0.075. The unit was run for overnight (20-22 hrs) with a constant supply of 18 volts per strip. After removing the strips from the unit, they were dried in oven at 100-110^{o}C for 1 hour. The strips were then immersed in bromophenol blue overnight . They were washed in 5% acetic acid 2-3 times, then dipped into fixative solution containing 300 mg Na-acetate in 5% acetic acid and dried in air. Each band was separated and eluted in 6 ml of 0.01 N NaOH for 30 minutes with constant shaking and the extinction was read at 540nm for determining the amount of protein in each band. The related percentage of different fractions of protein were calculated subsequently.

DETERMINATION OF PLASMA ASCORBIC ACID AND DEHYDROASCORBIC ACID BY 2,6 DICHLOROPHENOL- INDOPHENOL TITRATION

For determining plasma ascorbic acid level 4 ml of fresh plasma was mixed with an equal volume of 10% trichloracetic acid and filtered . 0.2 ml of .008% 2,6 - dichlorophenol indophenol was then titrated with the filtrate until the reddish colour disappeared. 0.2 ml of dye is equivalent to 8 μg of ascorbic acid (Varley, 1981). Amount of ascorbic acid present in the plasma was calculated from the volume of filtrate required for titration. To estimate the *dehydroascorbic acid content of the plasma*, dehydroascorbic acid was reduced back to ascorbic acid by bubbling hydrogen sulphide through the remaining part of the filtrate. Titration of 0.2 ml of 2,6-dichlorophenol indophenol was repeated again with the H_2S passed filtrate and the total amount of ascorbic acid and dehydroascorbic acid present in plasma calculated. The difference between the two results was then calculated to determine the amount of dehydroascorbic acid content of the plasma (Varley 1981).

Calculation

$$\text{Plasma ascorbic acid (mg/l)} = \frac{1000}{\text{ml titration}} \times 2 \times \frac{8}{1000}$$

$$= \frac{16}{\text{ml titration}}$$

SUMMARY OF EXPERIMENTAL PROTOCOL FOR THE DETERMINATION OF PLASMA ASCORBIC ACID AND DEHYDROASCORBIC ACID

4 ml of fresh plasma + 4ml of 10% TCA

Filtered

Filtrate

2 Equal Parts

ASCORBIC ACID ESTIMATION	DEHYDROASCORBIC ACID ESTIMATION
Filtrate as such	H_2S passed for 1-2 min. through the filtrate
Titrated with 0.2 ml of .008% 2,6 dichlorophenol indophenol	Dehydroascorbic acid reduced to ascorbic acid
Until the reddish colour disappear	Titration with 0.2 ml of .008% 2,6 dichlorophenol indophenol repeated
[Titration 1]	[Titration 2]
Volume of filtrate required for titration noted	Volume of filtrate required for titration noted

Amount of ascorbic acid in both Titration 1 and Titration 2 calculated

$$\text{Ascorbic acid mg/l} = \frac{1000}{\text{ml titration}} \times 2 \times \frac{8}{1000} = \frac{16}{\text{ml titration}}$$

Since 0.2 ml of 2,6 dichlorophenol indophenol is equivalent to 8 μg of ascorbic acid.

Result of Titration 1 = Amount of Plasma Ascorbic Acid

Result of Titration 2 - Titration 1 = Amount of Plasma Dehydroascorbic Acid

DETERMINATION OF LEUCOCYTE ASCORBIC AND DEHYDROASCORBIC ACID The leucocyte ascorbic acid and dehydroascorbic acid level was determined by 2,4 dinitrophenyl hydrazine method (Varley, 1981).5 ml of venous blood was collected in a diluent containing .85/NaCl,10% EDTA and 6% dextran,mixed and allowed to stand for 30 minutes.10 ml of the supernatant was then taken, mixed well and centrifuged for 15 minutes at 3000 rpm. After discarding the supernatant, 2.6 ml of 5% TCA was added to the compact button of leucocytes and platelets, homogenised thoroughly and centrifuged. Then three tubes were set in the following manner :

Tube No.1 : 1 ml of the supernatant obtained was taken for ascorbic acid estimation.

Tube No.2 : Hydrogen sulphide was passed through 1 ml of supernatant to reduce the dehydroascorbic acid present in the leucocytes back to ascorbic acid.

Tube No.3 : 1 ml of TCA was taken as blank.

Tube No.4 : 1 ml of 10 mg/l ascorbic acid was taken as standard.

To each tube was then added 0.3 ml of colour reagent composed of 2.2% 2,4- dinitrophenyl hydrazine, 5% thiourea and 0.6% copper sulphate (mixed in proportions 20:1:1) and all the tubes incubated at 37°C for 4 hours. After the incubation the tubes were cooled in ice-water and 2 ml of sulphuric acid added to each tube, mixed and the extinction read at 520 nm. The difference between the results of tube no.2 and tube no.

1 was then calculated to determine the dehydroascorbic acid content of leucocytes. A leucocyte count was carried out on the remaining of the original supernatant.

Calculation

Ascorbic acid content of supernatant (mg/1)

$$= \frac{\text{Reading of unknown}}{\text{Reading of standard}} \times \text{Conc. of standard.}$$

Amount of ascorbic acid in cells of buffycoat (leucocyte + platelet) as (μg/ 10^8 leucocytes)

$$= \frac{\text{Supernatant ascorbic acid (mg/l) x 2.6}}{\text{Leucocyte count/ } \mu\text{l supernatant}}$$

$$\text{Leucocyte ascorbic acid} = \frac{\text{Buffy layer ascorbic acid}}{\text{Conversion factor}}$$

The conversion factor is 2 with normal platelet and leucocyte count but varies with diseases such as thrombocytopenia and leukemia. Here factor 2 has been used for calculation.

QUANTITATION OF IMMUNOGLOBULINS BY SINGLE RADIAL IMMUNODIFFUSION METHOD Quantitation of immunoglobulins IgG and IgM were carried out with the help of Immuno Quanton Plate System which is based on Mancini's radial immuno diffusion technique (Span Diagnostics, India) (Mancini, 1965). 5 microlitres of diluted reference and test serum containing the immunoglobulin were applied into wells prepared in the

antibody-agarose plate with the help of a microcapillary that was supplied with the plate system.

The lids of the plates were then closed and the plates incubated at room temperature in a humid chamber. The incubation period for IgG plate was 48 hours, while that for IgM was 72 hours. After incubation, the diameter of each precipitin ring was measured accurately with the help of the measuring scale (Quantometer). The immunoglobulin concentration corresponding to the measured diameter were read directly from the reference value chart provided for the particular batch of Immuno Quanton Plate.

ASSAY OF T CELLS BY SHEEP ERYTHROCYTE ROSETTE FORMATION

Lymphocytes for the assay were obtained by layering 3 ml of blood on ficoll-hypaque gradient. The cell number was adjusted to 2 x 10^6 cells/0.2 ml suspension. Then to 0.2 ml cell suspension in a test tube containing 2 x 10^6 cells 0.2 ml of 1% SRBC and 0.2 ml 10% AB serum were added and incubated at room temperature for half an hour. After the incubation the suspension was centrifuged at 2000 rpm for 6 minutes and incubated again at 4-8^{0}C for half an hour. The cell pellet was then gently resuspended by rocking back and forth .0.2 ml trypan blue (0.2%) was then added slowly and the suspension observed under the microscope. Lymphocytes surrounded by three or more red cells were counted as

rosettes.Percentage of E-rosette was determined in reference to total lymphocytes (Chakraborty et al, 1981; Bauer, 1982).

NYLON WOOL COLUMN SEPARATION OF LYMPHOID CELLS Out of several techniques for separating the T and B lymphocytes, we opted for the nylon wool fibre column separation technique as outlined by Julius and co-workers (1973) because of its simplicity and rapidity and also because the cells are not exposed to any harsh treatment during this procedure.

About 100 mg of nylon wool was cut into small pieces and teased into loose fibres devoid of knots. The wool was then boiled in 1N HCl for 10 minutes to remove any toxicity. Then the nylon wool was washed for 3-4 times with triple distilled water to remove the acidity.The fibres were then washed first with 1(N) NaOH to neutralize the acidity and then again with triple distilled water (2-3 times).The washed wool was soaked overnight in a mixture of 0.2% EDTA and 0.2% $NaHCO_3$, washed in distilled water, dried and autoclaved.

The sterilised nylon wool was separated into small pieces, soaked in RPMI and packed into a 2 ml syringe. About 1 ml of cell suspension containing upto 6 x 10^6 cells was loaded carefully in the nylon wool column and incubated for 1 hour at 37^0C in a humidified atmosphere containing 5% CO_2. Then the nylon wool non adherent cells were eluted out with

an excess amount of warm RPMI and resuspended in fresh medium. The column was then filled with chilled RPMI and further incubated in ice for 10 minutes. Now the nylon wool adherent cells were eluted out with an excess amount of cold RPMI by vigorous agitation of the wool and then re-suspended in fresh medium. The cells were then counted with the help of a haemocytometer.

IN VITRO CULTURE OF LYMPHOCYTES WITH PHA The T-lymphocytes obtained from the nylon wool column were resuspended in RPMI 1640 (AT 060 , Hi-Media, Bombay), supplemented with penicillin-streptomycin (50 uml^{-1}),nystatin (50 uml^{-1}) and 10% AB serum. For activation of T cells two doses 5 and 10 μg of phytohemagglutinin (PHA) (Difco.Lab. U.S.A) in a volume of 5 μl and 10 μl were added in tubes containing 1 x 10^{6} cells in 1 ml culture medium. 0.2 ml of this suspension containing 2 x 10^{5} cells were then added to each well of culture plate.The plates were then incubated at $37^{o}C$ in a humidified atmosphere of 7.5 % CO_2 in air for 48 hours.

MEASURE OF BLASTOGENESIS After binding with the mitogen or antigen, metabolic activities of small lymphocytes get augmented and they gradually transform into bigger cells, known as blast cells. Thus blastogenesis is considered as one of the indicator of activation of lymphocytes and the percentage of blast cells in a lymphocyte population

stimulated with mitogen or antigen can be a measure of degree of activation of lymphocytes. Blast cells were counted by haemocytometer in presence of trypan blue under the microscope fitted with an occulometer. Cells with diameters greater than 7 μm were scored as medium sized and cells with diameter greater than 10-11 μm were scored as large-blasts. The proportion of blast cells was determined from the sum of viable medium plus large lymphocytes divided by the total viable lymphocytes counted (Chakravarty & Clark, 1977).

MEASURE OF DNA SYNTHESIS DNA synthesis of the proliferating T cells was measured by the degree of incorporation of ^{3}H-thymidine. Twelve hours prior to the time of harvesting 0.5 μCi of ^{3}H-TdR (Sp. Act 18.5 Ci/mM, BARC, Bombay) was added per well of the culture plate containing 2 x 10^{5} cells. At the end of the incubation period the T cells were first washed with PBS on small filter paper discs (Whatman filter paper no. 3) under suction pressure, then with 10% TCA and again with PBS. The filter paper discs were dried and kept in standard scintillation vials for counting. An amount of 5ml scintillation fluid was added to each vial before counting in the scintillation counter (Beckman LS 1800). The scintillation cocktail was prepared by adding 6 gm PPO and 0.5 gm POPOP in 1 litre toluene.

CYTOTOXICITY ASSAY Cytotoxic ability of T lymphocytes was determined by using ^{51}Cr release assay (Dunklay et al, 1979; Thorn et al, 1979). This assay is based upon the finding that radioactive chromium ions ($^{51}Cr_3O_4^-$) diffusing into a cell are retained in the cytoplasm for a considerable period of time. This internal ^{51}Cr is released into supernatant fluid following cell membrane damage caused by cell mediated cytotoxic response of the effector lymphocytes. To obtain the target cells, the method of Melief et al (1979) was adopted. The target cells were blast cells from allogeneic source generated in vitro by PHA stimulation. Following the method outlined earlier the blastoid cells were generated in vitro by stimulating lymphocytes from another person with 10 μg/ml PHA for 48 hours. The allogeneic target cells thus obtained were labelled with $Na_2{}^{51}CrO_4$ (Sp. Act.50 mCi/mg, BARC, Bombay). An amount of fluid containing 200 μci $Na_2{}^{51}CrO_4$ was added in 1 ml of cell suspension containing 10^7 cells and the cells were incubated for one and a half hour in a humidified atmosphere of 7.5% CO_2 in air at 37^{o}C . The tubes containing the cells were shaken 3 times during incubation for facilitating the labelling. After incubation the cells were washed three times with PBS and the number of cells was adjusted to 4 x 10^4 cells/ ml. Effector cells were obtained from the alcoholics and the cell number adjusted to 1 x 10^6 cells/ml.

To 1 ml of effector cells, 250 μl of ^{51}Cr labelled

target cell suspension was added in 3 different ratios of 100:1, 50:1 and 10:1 and the mixture was incubated for 6 hours. Effector cells were not added in the control culture for spontaneous release of isotope from labelled target cells. 1 ml medium was added in each tube instead and the tubes were also incubated for 6 hours. For maximum release of radioactivity from target cells 1 ml of distilled water was added instead of medium in the tubes containig labelled target cells only. After incubation, culture tubes were centrifuged except the tubes for maximum release of radioactivity. 1 ml supernatant was collected from each tube and the amount of ^{51}Cr-release into the supernatant was assessed by using a gamma ray spectrometer (Model No. GR523A, ECIL, India). The percentage of cytotoxicity of ^{51}Cr release by the effector cells was calculated as follows:

$$\frac{\text{Experimental release - Spontaneous release}}{\text{Maximum release - Spontaneous release}} \times 100$$

The cytotoxicity index has been calculated on the basis of triplicate value in an experiment and each experiment has been repeated thrice.

ASCORBIC ACID AND ZINC THERAPY After the initial tests the alcoholics were divided into two groups of 6 subjects and treated separately with ascorbic acid and zinc sulphate. As the alcoholics who were treated with ascorbic acid or zinc

acted as self controlled subjects, the effect of some other superimposed illness would be ignored obviously. Still care was taken to see that the subjects selected for the study were not suffering from any other chronic or acute illness which might have affected the hepatic function as evidenced by priliminary biochemical and immunological test.

Ascorbic Acid Theraphy Ascorbic acid is known to be not only a potent detoxicant but also an effective radical scavenger.Therefore mega ascorbic acid therapy was introduced in the ist group of alcoholics and the effect observed on all the parameters studied before therapy. The subjects were treated orally with 3 grams of ascorbic acid per day in three divided doses. The treatment continued for a month. This megadose was found to be nontoxic to human bodies in our laboratory, with normal renal function and normal urinary oxalate output. (Unpublished Observations).

Zinc Therapy Besides ascorbic acid zinc has also been used for therapy as there are some recent reports indicating its detoxicant and immunostimulant function. Thus the second group of alcoholics were supplemented with oral zinc sulphate for a month. Zinc sulphate was used at a dose a 660 mg per day in three divided doses as practised by the clinicians as a therapeutic measure . After the treatments, the different estimations were made again according to standard protocols as outlined earlier.