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CONTENTS

Effects of chemical industry effluents on humoral immune response in mice

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ABSTRACT: Effluents from chemical industries are released abundantly into the environment many times without proper treatment. Such effluents find their way into the surrounding water bodies and thereby making water unfit for human and animal consumption. Limited investigations have been carried out on the impact of chemical industry effluents on health of animals and men. Therefore, present study was done to study the effect of chemical industry effluent on humoral immune response in mice. To study the effect of chemical industry effluent on humoral immune response, two-weeks-old 256 mice procured from Indian Veterinary Research Institute, Izatnagar, Bareilly were randomly divided into four equal groups of 64 mice each viz. control (group-1), R2B vaccine + effluent treated (group-2), effluent treated (group-3) and R2B vaccinated (group-4). The vaccine used was R2B strains given to group-2 and 4 @ 0.1 ml by intraperitoneal route. Humoral immune response was assessed by Lymphocyte Stimulation Test using LPS and by performing ELISA at 15th, 30th,45th,60th,75th,90th,105th and 120th day post treatment DPT. The mean ΔOD and antibody titer was significantly lower in group 2 and group 3 mice on 90, 105 and 120 days of observation in comparison to groups 1 and 4 mice. These findings clearly suggest that the metal exposed animals might have a decreased ability to develop humoral immune responses.

Key words: Chemical industry effluents, humoral immune response, mice

In the modern days of industrialization, the word 'pollution' is more often affiliated with 'environment'. In the last few decades, the concerns regarding the environmental pollution have been raised more than ever before. It has become a major global problem, resulting in deterioration in the quality of life, air, water and soil, thereby posing threat to human and animal health. Chowdhury and Naha (2002) reported that magnitude of environmental pollution has increased many folds during the 20th century with rapid industrialization and expansion of mechanization in the industrial sector. Effluents from chemical industries are released abundantly into the environment many times without proper treatment. Such effluents find their way into the surrounding water bodies and thereby making water unfit for human and animal consumption. The residues of the chemicals persist in various food stuffs like cereals, grains, fodder, egg, meat and milk. The heavy metal residues are not only harmful to animals but also causing serious disorders in man by injuring target organs like liver, kidney or immune systems. (Koller, 1973). The alterations in the immune system may result in lower immune competance of an individual leading to vaccine failures, occurrence of outbreak of various diseases. (Koller *et al*., 1975). These effluents causing alteration in the health, which may be because of alteration in humoral immune response. (Koller *et al.*,1976). Keeping in view the nature of chemical industry effluent, a survey was conducted around chemical industries. The impact of such effluent on humoral immune response was studied using a mice model.

MATERIALS AND METHODS

To study the effect of chemical industry effluents, the samples of effluent were collected from and nearby effluent passing areas through the naala near Jubilant organosys (Gajraula) and brought to the laboratory. The toxicity of the effluent was studied in laboratory animals (mice) by giving effluent water *ad libidum* for four months of duration.

For this study, 256 mice of 2 weeks age were procured from IVRI, Izatnagar, Bareilly. Before start of experiment, the experimental house was thoroughly cleaned with water and 1% phenyl

solution. Cages and utensils were washed with water and phenyl solution and cleaned with potassium permanganate solution. Total 256 mice were randomly divided into four groups of 64 mice each viz., control (group-I), Effluent +R2B vaccinated (group-2), effluent treated (group-3) and R2B vaccine treated (group-4). The vaccine used was R2B strains and was given to group-2 and 4 @ 0.1 ml by intraperitoneal route. For assessment of humoral immunity, blood was collected and serum was separated.

ELISA was performed by single dilution method as per the procedure described by Chandrashekhar (1994). The appropriate concentration of viral antigen used in the test was determined by checkerboard titration. The antigen was diluted 1:80 in carbonate bicarbonate buffer (pH 9.6) and 100 µl of this diluted virus was added to each well of 96 well ELISA plate (NUNC). The plate was incubated over night at 4°C. Next day, antigen coated plate were washed thrice with PBS-T for five minutes each and tapped thoroughly. The unreacted sites were blocked by adding 200 µl of 5% skimmed milk powder (LOVA-chemical) to each well of the plate. The plates were incubated for 2 hours at 37°C. The plates were again washed as mentioned above. The single dilution of the test serum was made in eppendorf tubes by adding 2 µl of test serum and 200 µl of PBS-T per tube resulting into dilution of 1:100. Positive control serum (against local isolate of R_2B virus) and negative serum were also diluted 1:100 in PBS-T. The diluted negative control serum was added in 100 µl quantity to the first three wells of the first row and 100 µl of diluted positive serum was added to the first 3 wells of the first row and 100 µl of diluted positive serum was added to the last 6 wells of last row of ELISA plate and last three wells of the last row kept as blank. In remaining wells of plate, 100 µl of dilute test serum was transferred to each of the three corresponding wells. The plate was incubated at 37°C for 2 hours and washed thrice with PBS-T for five minute each ad tapped thoroughly. Then 100 µl of rabbit anti-mouse horse radish peroxidase conjugate (Sigma) diluted 1: 800 in PBS-T (Chauhan, 1998) was added to each well and plates were incubated at 37°C for 2 hours. Each well of the plate was then added with 100 µl of freshly prepared substrate buffer ortho-phenylenediamine dihydrochloride (OPD) in citrate buffer followed by H_2O_2 and then incubated at 37°C for 30 minutes in dark. The reaction was stopped by adding 100µl of stop solution (H_2SO_4) in each well of plate. The plate was read at 492 nm in a ELISA reader.

Calculation of ELISA titer

The average absorbance of positive and negative control was calculated from the absorbance value of ELISA plate and corrected positive control (CPC) value was determined subtracting average negative absorbance from average positive absorbance.

The specific value (Sp. Value) was calculated using following formula:

$$\text{Sp. Value} = \frac{\left[\text{Average absorbance of test sample}\right] - \left[\text{Average absorbance of negative control}\right]}{\text{Corrected positive control}}$$

The titer was then calculated by $\log_{10}$ titer = [1.464 x $\log_{10}$ Sp.] + 3.197

Titer = Antilog of $\log_{10}$ titer

Lymphocyte Stimulation Test

B lymphocyte blastogenesis assay was carried out as described by Rai-el Balhaa *et al*. (1987) with some minor modification according to Chauhan (1998) using RPMI-1640 as test medium and Lipopolysaccharide (LPS) as mitogens for B lymphocyte. The reduction of the MTT dye (3-[4, 5-dimethylthiazol-2-Y]-2-5 diphenyltetrazolium bromide) to formazon was used as an indicator of cell proliferation.

Isolation of Lymphocytes

Spleen was collected from experimental mice under aseptic conditions and cut into small pieces and suspended in RPMI-1640 medium. Suspension was filtered through sterile muslin cloth and cells were counted in filtrate using Trypan blue (0.5 %) dye exclusion test. Finally, the lymphocyte count was adjusted to10^6 cells/ml in RPMI-1640 medium.

Procedure

Triplicate cultures were made using 100 µl of medium alone or medium containing LPS in flat bottom sterile micro filter plate (TPP Europe). The plate was sealed with the tape and incubated for three days at 37°C in CO_2 incubator. Total 50 µl of MTT dye (3-(4, 5-dimethlthiazol-2-Y)-2-5 diphenyltetrazolium bromide) was added to all the wells and the plate was again sealed and incubated for another four hours at 37^0C in CO_2 incubator. To each well 100 µl of acid isopropanol was added and the absorbance of each well was determined in microscan ELISA reader at wave length of 570 nm. An average value of triplicate wells was taken and the mean optical density of mitogens stimulated culture was obtained. The mean O.D. value of control well was subtracted from mean O.D. of well with mitogen and presented as ΔOD.

RESULTS AND DISCUSSION

The mean ELISA values reflecting the effects in group 2 and humoral immunity up to 120 days were recorded at the interval of 15 days and are presented in Table 1 and figure 1 along with group 4 for comparison. The mean ELISA values at 15th day in group 4 and 2 were 0.137±0.008 and 0.136±0.009, respectively. The mean ELISA value of group 2 varied non-significantly up to 75th day. However, the antibody titer decreased significantly on 90th day (P0.05), 105th day (P0.05) and 120th day (P0.05) as compared to ELISA titer of group 4 and values were 0.116±.0.007, 0.110±0.006 and 0.108±0.008, respectively. The mean ΔOD of lymphocyte culture of different groups using lipopolysaccharide (LPS) as mitogen is presented in Table 2 and Figure 2. The mean ΔOD values in group 2 and group 3 were significantly lower than groups 1 and 4 during the investigation period. The mean ΔOD values in group 2 at 15 day was 0.190±0.004 and it decreased significantly on 90th day (p d" 0.05) 105th day (p d" 0.05), and 120th day (pd" 0.05) as compared to the values in groups 1 and 4 on these days. Its values in group 2 were 0.160 ±0.004, 0.159±0.006 and 0.156±0.006, respectively on 90,105 and 120 DPT. The mean ΔOD values in group 3 at 15th day was 0.180±0.004 which decreased significantly on day 90 (p d" 0.05), 105th day (p d" 0.05) and day 120 (pd" 0.05) as compared to groups 1 and 4 on these days. Its level in group 3 on days 90, 105 and 120 were 0.143± 0.006, 0.140±0.007 and 0.131±0.003, respectively.

In the present study, the mean antibody titer of group 2 mice declined non-significantly up to 75 days of observation. However, from 90th day up to 120th day of observation, the antibody titer decreased significantly in group 2 mice when compared to increased ELISA titer on all days of observations in vaccine treated mice of group 4. The above findings are in agreement with the finding of Upadhayay (2007) who also observed decreased antibody titer in mice fed with paper and pulp industry effluent. Heavy metals are a prominent class of immunotoxins associated with both specific and non-specific immunoenhancement and immunosuppression. As a result of this ability to alter immune system homeostasis, some metals like cadmium and lead have been implicated as causative agents or aggravating factors in the development of chemical hypersensitivity, allergy and autoimmune disease or in increased susceptibility to infections (Bigazzi, 1999; Dayan, 1990; Druet, 1995; Lawrence and McCabe, 2002 and Peden, 2002). Some results also suggested immuno- suppressive role of both these metals on immune function (Cook *et al.*, 1975; Ewers *et al.*, 1982; Fujimaki *et al.*, 1983; Exon *et al.*, 1986; Descotes, 1992; Fischbein *et al.*, 1993; Ritz *et al.*, 1998 and Sarasua *et al.*, 2000). Immunosuppressive effects of low dose of cadmium feeding in laboratory animals were earlier observed by Koller (1973). Koller *et al.* (1975) found that subclinical doses of cadmium produce a significant decrease in antibody (IgG) synthesis, an indicator that the memory cells or T-helper cells were adversely affected. The suppression of humoral immune response was also observed by Chauhan and Agrawal (1999) in mice given chronic low doses of cadmium for 4 months. The low antibody titer in the heavy metal rich effluent exposed mice could be used to explain the increased sensitivity of animals to infections (Truscott, 1970).

Environmental pollution as a result of industrial wastes has lead to increased levels of heavy metals

Table 1: Humoral immune response (antibody titer) in experimental mice through ELISA against R_2B virus antigen (Mean value ± SE)

Days of Observation		30	45	60	75	90	105	120	15
Mean ELISA Titer	Vaccine treated (Gp. 4)	0.137± 0.008	0.139± 0.008	0.140± 0.009	0.142± 0.005	0.145± 0.009	0.150± 0.002	0.155± 0.009	0.160± 0.001
	Effluent+vaccine treated (Gp. 2)	0.136± 0.009	0.134± 0.009	0.130± 0.008	0.120± 0.008	0.116± 0.007*	0.110± 0.006*	0.108± 0.008*	0.132± 0.009

*represents significant difference between means of different groups at P<0.05

Table 2. Humoral immune response in experimental mice through Lymphocyte Stimulation Test (LST) using LPS (Mean ΔOD ± SE)

Days of Parameters	Control (Gp-1)	Effluent+ vaccine treated (Gp-2)	Effluent treated (Gp-3)	Vaccine treated (Gp-4)
15	0.113±0.007	0.190±0.004	0.180±0.004	0.195±0.005
30	0.182±0.001	0.180±0.005	0.171±0.003	0.205±0.011
45	0.243±0.005	0.175±0.003	0.165±0.004	0.250±0.0047
60	0.218±0.009	0.170±0.003	0.184±0.004	0.248±0.0047
75	0.204±0.008	0.169±0.003	0.155±0.007	0.331±0.004
90	0.216±0.009	0.160±0.004*	0.143±0.006*	0.336±0.004
105	0.230±0.006	0.159±0.006*	0.140±0.007*	0.292±0.007
120	0.225±0.008	0.156±0.006*	0.131±0.003*	0.325±0.004

*represents significant difference between means of different groups at P<0.05

in ecosystem and food chain. Heavy metals such as lead, cadmium, mercury, etc. present in soil, water, air and feed items as contaminants may exert their deleterious effect on the immune system of animals as well as human beings (Koller, 1979). The lowered immune competence in animals due to environmental pollutants may cause increased susceptibility to various infections, occurrence of re-infection, epidemics of diseases and vaccination failures. All living creatures have a defense mechanism (immune system), which protects the body from different infections. In the past decades, there have been reports of vaccination failures and disease outbreaks even after proper vaccination which may be due to harmful effects of environmental pollutants on the immune system of animals (Chauhan, 1995). According to Karnik (2001), evaluation of cell proliferation and function, appears to be more sensitive than measurement of cell viability to determine the effects of heavy metals. It is suggested that lead and cadmium bind to sulphydryl alkyating agents and modulate the membrane bound thiols thus altering the function. The spleen is the main secondary lymphoid organ, where mature and immunocompetent lymphocytes are present, intermingled with cells of the mononuclear phagocyte system, and immunity responses against the antigen stimuli take place here. Its function is to eliminate antigens from the blood stream. Approximately, 50% of all lymphocytes that migrate through the body passes through the spleen (Pabst, 1988), which is an important place for the induction of immune effective responses (Biuting *et al.,* 1996). The dysfunction of the spleen alters many immunological parameters such as the number

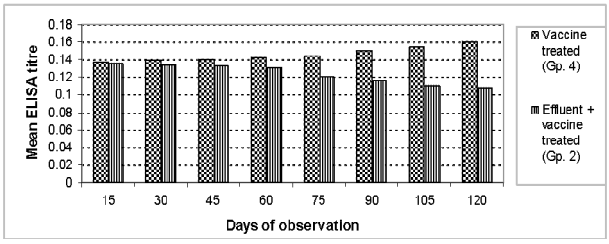

Fig. 1: Determination of humoral immune response (antibody titer) in experimental mice through ELISA against R_2B virus antigen

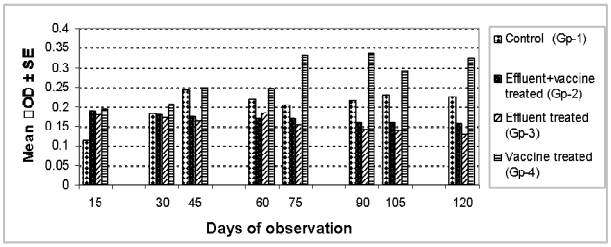

Fig. 2: Humoral immune response in experimental mice through Lymphocyte Stimulation Test (LST) using LPS

of B-lymphocytes (antibody secreting cells) in blood (Westermann *et al.,* 1990) and it can enhance susceptibility of animals to various bacterial infections.

In the present study, humoral immune responses were assessed in mice by lymphocytes stimulation using LPS. On employing MTT assay, the mean ΔOD of lymphocyte culture of different groups using lipopolysaccharide (LPS) as mitogen were significantly lower in effluent + vaccine treated (group 2) and effluent treated (group 3) mice on 90, 105 and 120 days of observation in comparison to groups 1 and 4 mice. Decrease in the lymphocyte proliferation with LPS is an indication of the suppression of B cell blastogenesis which is essential for humoral immune responses. These findings clearly suggest that the metal exposed animals might have a decreased ability to develop humoral immune responses. These observations are consistent with the previous studies demonstrating the immunosuppressive effects of heavy metals. The immunosuppressive activity of the heavy metals have been correlated with their toxic potential and inhibition of lymphocyte proliferation (Homberger *et al.,* 1983) and/or by stimulating synthesis and secretion of adrenal corticosteroids which in turn suppress the antibody forming cells (Cheville, 1978). Chauhan and Agrawal (1999) also recorded significant decrease in T-lymphocyte and B-lymphocyte blastogenesis in cadmium fed calves given 10 mg/kg body weight cadmium in drinking water for 4 months. The decreased ability of lymphocytes to develop a blastogenic response might be a factor in the decreased resistance to infection and decreased response to treatment of animals given heavy metals (Haggard *et al.,* 1980). Shabani and Rabbani (2000) concluded that lead exposure in animals damages lymphocytes by oxidative stress. According to Mc. Cabe *et al.* (1991), T-lymphocytes seemed to be a target of lead immunotoxicity and lead tended to decrease lymphocyte proliferation by LPS while, cadmium stimulated blastogenesis.

CONCLUSION

Chemical industry effluent causes immunosuppression on animals and therefore, effluent should be released only after proper treatment to prevent immunosuppression in the animal and human being.

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