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Immune Functions of Former Poison Gas Workers
I. Mitogenic response of lymphocytes and serum factors

Michio YAMAKIDO, Jitsuro YANAGIDA, Shinichi ISHIOKA, Shigeru MATSUZAKA, Soichiro HOZAWA, Masatoshi TAKAISHI, Tsutomu INAMIZU, Mitoshi AKIYAMA* and Yukio NISHIMOTO

The Second Department of Internal Medicine, Hiroshima University School of Medicine, 1-2-3, Kasumi, Minami-ku, Hiroshima 734, Japan
** Department of Immunology, Radiation Effects Research Foundation, Hiroshima 730, Japan*
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ABSTRACT

The relation of depressed immune function to carcinogenesis has been estimated in the living body. The authors have measured the immunological parameters in former poison gas workers, a group having a high risk of carcinogenesis, for comparison with age matched normal controls and the following results were obtained.

1) With regard to serum factors, no significant difference could be demonstrated between normal controls and poison gas workers in such immunoglobulins as IgG, IgA, and IgM, in acute phase reactants such as α_1-AT, α_1-AG, α_2-HS and C_3 and in such tumor markers as CEA, ferritin, and β_2-microglobulin. Furthermore, no difference could be observed in the positive rate of immune complex and in complement activity.

2) No difference could be observed between the two groups with regard to tuberculin skin reaction and number of lymphocytes, but the longer the duration of work at the poison gas factory, the more significant was the increase in those who showed negative tuberculin skin reaction.

3) In comparison with normal controls, mitogenic response to PHA showed a significant decrease in poison gas workers, but no significant difference could be seen in mitogenic response to Con A and PPD and in mixed lymphocyte reaction.

4) No significant difference could be demonstrated between the two groups in the inhibitory effects of serum on mitogenic response to PHA and Con A and on mixed lymphocyte reaction.

On Okunojima, an island lying off the coast of Tadanoumicho, Takehara City in Hiroshima-Prefecture, was located a poison gas manufacturing factory of the former Japanese Army, which was engaged during World War II in the manufacture of such erosive and highly lethal gases as yperite (sulphur mustard) and lewisite together with sneezing gas, tear gas and asphyxiating gas[21].

Since 1952 and for more than 30 years, the Second Department of Internal Medicine of Hiroshima University School of Medicine has been engaged in a health survey of the former workers of this poison gas factory (poison gas workers). In this group not only a high incidence of chronic lung diseases such as chronic bronchitis[16] but also of respiratory tract neoplasms has been observed and the frequency has reached as high as 37-fold that of the incidence of respiratory tract neoplasms in

Japan[17,22,23]. Furthermore, in recent years there has been an elevated incidence of malignancy of the digestive system such as gastric cancer and hepatoma and also of skin cancer. Also, the frequency of double cancer has reached as high as 5-fold that of the general population and cases of triple cancer have also been seen in this group. As for their cause, Nishimoto et al[17] in focussing their interest to the highly carcinogenic yperite gas have reported that the greater the opportunity which the type of work required contact with yperite gas and the longer the duration of such work, the higher was the incidence of cancer.

Since 1970 when Burnet[3] first proposed the concept of "immunological surveillance" interest has been focussed on the relation of depressed immunity to carcinogenesis and extensive analysis have been conducted. These studies have shown that various immunological abnormalities are present in tumor-bearing hosts.

The authors on the assumption that delineation of immunity in these poison gas workers would provide a model for elucidating the process leading to carcinogenesis have measured various immunological parameters in poison gas workers and age matched normal controls.

In the present study, measurements were made on such immunoglobulins (Ig) as IgG, IgA, and IgM, on acute phase reactants such as α_1-antitrypsin (α_1-AT), α_1-acid glycoprotein (α_1-AG), α_2-heat stable glycoprotein (α_2-HS), and C_3, on tumor markers such as carcinoembryonic antigen (CEA), ferritin and β_2-microglobulin (β_2-M), and on immune complex and complement activity for serum factors; on tuberculin skin reaction for delayed type hypersensitivity (DTH); on number of peripheral blood lymphocytes (PBL) for the number of immunocytes; on mitogenic response to phytohemagglutinin (PHA), concanavalin A (Con A) and purified protein derivative (PPD) and mixed lymphocyte reaction (MLR) for T cell function; and on the inhibitory effects of serum on these T cell functions. The results will be reported.

SUBJECTS

The subjects of the present study are 118 males composed of 46 normal controls and 72 poison gas workers. Their background is presented in Table 1. Healthy aged volunteers whose age distribution and smoking history are almost identical to the poison gas workers as shown in Table 1 were employed as normal controls. All poison gas workers had chronic bronchitis satisfying the definition given by Fletcher. Their mean estimated duration of chronic bronchitis is as shown in Table 1. The poison gas workers employed in the present study were not under the administration of steroids, antibiotics and antiinflammatory agents, and had not experienced acute aggravation of chronic bronchitis during the last three months and had not received X-ray examination during the last month. The type of work was classified according to the period of contact with mustard gas into groups A, B, and C. Workers who had been

Table 1. Background factors of the subjects

		Normal controls	Poison gas workers
Number of cases		46	72
Age (years old)		68.4 ± 8.2	68.3 ± 6.8
Sex		all male	all male
Clinical state		no clinical abnormality	all chronic bronchitis
Smoking	smoker	29 (63.0%) (707 ± 404)*	41 (56.9%) (642 ± 245)
	ex-smoker	6 (13.0%) (608 ± 486)	12 (16.7%) (598 ± 391)
	non-smoker	11 (24.0%)	19 (26.4%)
Type of work**	group A	—	26 (36.1%)
	group B	—	24 (33.3%)
	group C	—	22 (30.6%)
Duration of work***	-2 years	—	25 (34.7%)
	2-5 years	—	28 (38.9%)
	5- years	—	19 (26.4%)
Duration of chronic bronchitis (years)		—	18.9 ± 11.9

* Mean value of Brinkman's index.
** Numbers of poison gas workers by type of work in the poison gas factory.
*** Numbers of poison gas factory by duration of work in the poison gas factory.

directly engaged in production of mustard gas were classified into group A; workers who had been engaged in laboratory work, repair, and incineration and who might have had less opportunity of contacting mustard gas than group A were classified into group B; and workers who had had little opportunity to be in contact with mustard gas, that is, those who had been engaged in the production of gases other than mustard gas (that is, sneezing gas, tear gas and others), medical doctors, and clerical workers, were classified into group C. Furthermore, the duration of work was classified into three groups, that is, less than 2 years, 2 to 5 years, and more than 5 years. The details are shown in Table 1.

METHODS

Measurement of serum proteins

Quantitative analysis of such immunoglobulins as IgG, IgA and IgM and such acute phase reactants as α_1-AT, α_1-AG, α_2-HS, and C_3 was made by single radial immunodiffusion method[12], using antiserum and standard serum (Behring Werke). In the measurement of tumor markers, EIA kit (Abbott) for CEA and ferritin and RIA kit for β_2-M (Pharmacia) were employed.

Detection of circulating immune complex

Detection of immune complex was made by Clq solid-phase EIA method[15]. One ml of Clq solution adjusted to 3.0 μg/ml by phosphate buffer saline (PBS) is placed in a polystyrene tube and incubated for 17 hours at 4°C to prepare Clq coated tube. Sample serum diluted threefold in advance by 0.2 M EDTA is incubated for 30 min at 37°C in order to remove endogenous Clq. After diluting 30-fold with PBS, 1 ml is removed and placed in Clq coated tube and incubated at room temperature for 2 hr. The peroxidase activity bound to the tube which was treated with horse radish peroxidase labelled anti-human IgG antibody (Cappel) and washed is determined at the wave length of 510 nm with 4-aminoantipyrine and hydroquinone methyl ether as substrate. The concentration of immune complex of the sample serum is expressed as equivalent concentration to heat-aggregated human IgG (63°C, 15 min) and value greater than 1.5 μg/ml is evaluated as positive.

Detection of complement hemolysis activity

Detection of complement activity was made by 50% hemolysis method (CH_{50} method) of Mayer[13].

Sheep red blood cell (SRBC) suspension adjusted to 1×10^9/ml and hemolysin are mixed at equal volume at a concentration not permitting SRBC aggregation and after 10 min at 37°C this is incubated for 15 min at 0°C to prepare sensitized SRBC (EA). Sample serum diluted to an appropriate concentration is added to 5×10^8 EA to which is added gelatin veronal buffer to make the volume 7.5 ml. After incubated for 60 min at 37°C, it is centrifuged at 0°C and the absorbance of the supernatant is measured at a wave length of 541 nm. The hemolysis rate is computed and the dilution multiple of sample serum for 50% hemolysis is designated as the complement activity (CH_{50} unit/ml).

Measurement of tuberculin skin reaction

0.1 ml of PPD solution (Japan BCG) is intradermally injected in the forearm and after 48 hr the erythema is measured. The tuberculin reaction is then expressed as the mean of the longitudinal diameter and transverse diameter and value less than 10 mm is evaluated as negative.

Calculation of number of peripheral blood lymphocytes

The percentage of lymphocytes obtained from the blood smear multiplied by the number of peripheral blood leukocytes is designated as the number of PBL.

Assay of mitogenic response of lymphocytes

1.25×10^5 of PBL separated by Ficoll-Hypaque density gradient centrifugation method is suspended in 0.2 ml of Eagle minimum essential medium containing 10% heat-inactivated human AB serum and 1% non-essential amino acid (NEAA, Gibco) (complete MEM) and then added to 96 well microplate (Nunc). And then, to each well addition is made so that the final concentration will be 1% for PHA (HA-15, Wellcome), 20 μg/ml for Con A (Sigma), and 10 μg/ml for PPD (CIP, Japan BCG) and then cultured for 4 days at 37°C in 5% CO_2. At 24 hr prior to completion of culture, 0.4 μCi of ^{3}H-thymidine (^{3}H-TdR, 5 Ci/mmol, Radiochemical centre, Amersham) is added and upon completion of cul-

ture, PBL will be collected onto glass fiber filter with the use od automatic cell harvester (Labo Science). Incorporation of ^{3}H-TdR in PBL is measured by liquid scintillation counter and mitogenic response of lymphocytes is expressed as count per minute (cpm).

Assay of one-way MLR

PBL obtained from eight healthy donors are mixed at the same number and 1.25×10^5 of these PBL exposed to 1250 rad of cobalt are suspended in 0.2 ml of complete MEM (stimulator cells) and added to microplate. And then, to each well 1.25×10^5 of PBL obtained from the subjects (responder cells) are added and cultured for seven days at 37°C in 5% CO_2. At 24 hr prior to completion of culture, 0.4 μCi of ^{3}H-TdR is added and as described above incorporation of ^{3}H-TdR is measured. One-way MLR is expressed as cpm.

Determination of inhibitory effect of serum on mitogenic response and MLR

1.25×10^5 of PBL obtained from one healthy donor are suspended in 180 μl of Eagle MEM containing 1% NEAA but not containing serum and then added to each well of the microplate. As described above, PHA, Con A and stimulator cells of one-way MLR are added. Serum obtained from the subjects (sample serum) and serum obtained from PBL donor (control serum) are inactivated for 30 min at 56°C, added to each well at 20 μl and then cultured for the given time at 37°C in 5% CO_2. The response of each was obtained as cpm. The inhibitory effect of serum is obtained from the following equation.

$$\% \text{ inhibition} = \left(1 - \frac{\text{cpm of cultures with sample serum}}{\text{cpm of cultures with control serum}} \right) \times 100\ (\%)$$

In the present study assays using PBL were all conducted in triplicate, all the data are expressed as mean ± standard deviation (SD), and the statistical analysis was made by Student's t test. In results, normal control is abbreviated NC and poison gas workers PG.

RESULTS

Serum factors in poison gas workers

Immunoglobulins are shown in Fig. 1. IgG was 1,794 ± 467 mg/dl in NC and 1,698 ± 377 mg/dl in PG; IgA was 358 ± 145 mg/dl in NC and 424 ± 199 mg/dl in PG; and IgM was 149 ± 39 mg/dl in NC and 139 ± 50 mg/dl in PG, indicating that IgA was a slightly higher in PG, but the difference was not significant. Acute phase reactants are shown in Fig. 2. α_1-AT was 308 ± 50 mg/dl in NC and 305 ± 59 mg/dl in PG; α_1-AG was 75.7 ± 22.9 mg/dl in NC and 70.5 ± 19.9 mg/dl in PG; α_2-HS was 59.5 ± 8.5 mg/dl in NC and 58.7 ± 9.4 mg/dl in PG; and C_3 was 65.6 ± 15.7 mg/dl in NC and 68.0 ± 11.8 mg/dl in PG with no difference being demonstrated between the two groups. Tumor markers are shown in Fig. 3. CEA was 2.55 ± 1.33 ng/ml in NC and 2.94 ± 1.42 ng/ml in PG; ferritin was 131 ± 125 μg/ml in NC and 117 ± 119 μg/ml in PG; and β_2-M was 2.12 ± 0.77 μg/ml in NC and 1.97 ± 0.83 μg/ml in PG with CEA being slightly higher in PG, but the difference was not significant. Immune complex is shown in Fig. 4. The number of positive cases was 3 out of 16 cases (18.8%) in NC and 9 out of 72 cases (12.5%) in PG with no difference being demonstrated between the two groups. Complement activity (CH_{50}) is presented in Fig. 5. Complement activity was 35.5 ± 9.8 U/ml in NC and 35.5 ± 8.9 U/ml in PG with no difference being observed between the two groups.

Tuberculin reaction and number of lymphocytes in poison gas workers

Tuberculin reaction is shown in Fig. 6. The number of negative cases was 11 out of 46 cases (23.9%) in NC and 22 out of 72 cases (30.6%) in PG with the mean value being 16.5 ± 14.0 mm in NC and 15.2 ± 11.5 mm in PG. The number of negative cases was slightly higher in PG, but the difference was not significant. The number of lymphocytes is shown in Fig. 7. The number was 2,277 ± 1,025/mm^3 in NC and 2,546 ± 977/mm^3 in PG. The number was slightly higher in PG, but the difference was not significant.

Mitogenic responses and MLR in poison gas workers

Fig. 8 shows PHA response, The response was 28,140 ± 10,901 cpm in NC and 21,595 ± 6,607 in PG with a significantly low value being observed in PG. Con A response, PPD response

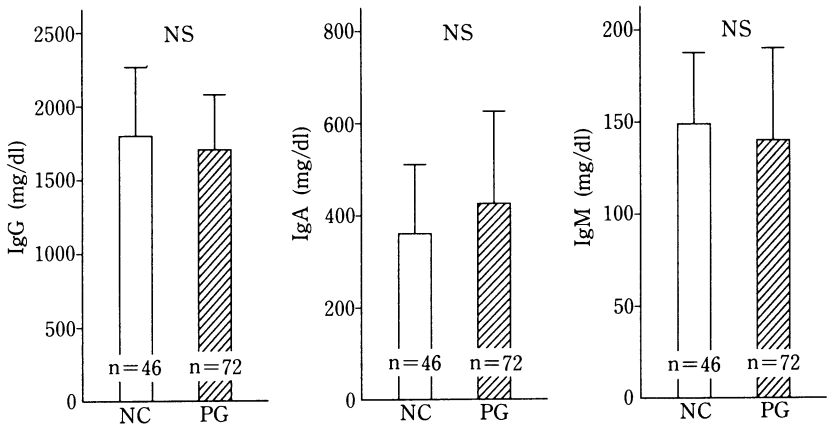

Fig. 1. Immunoglobulins in normal controls (NC) and poison gas workers (PG). Results are mean values ± SD.

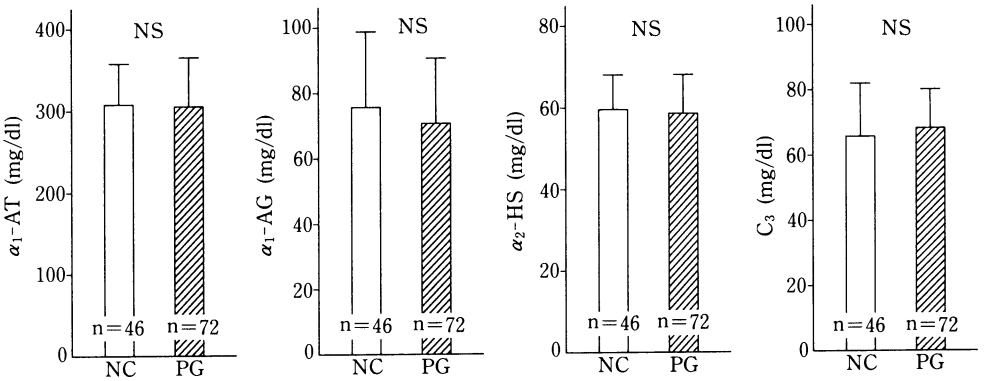

Fig. 2. Acute phase reactants in normal controls (NC) and poison gas workers (PG). Results are mean values ± SD.

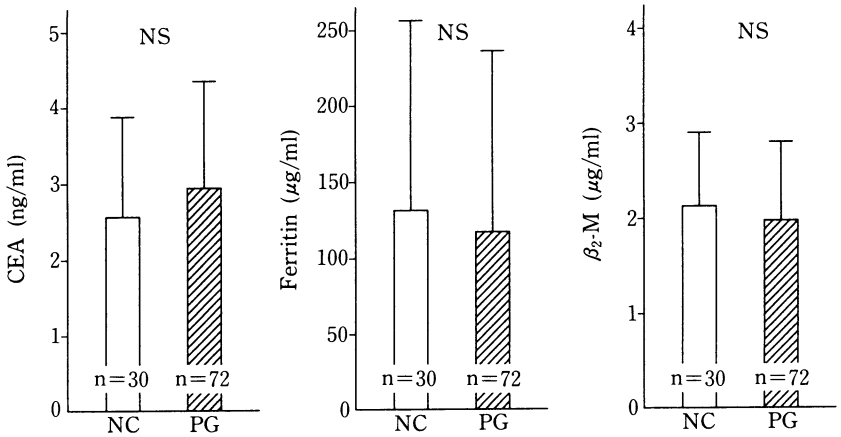

Fig. 3. Tumor markers in normal controls (NC) and poison gas workers (PG). Results are mean values ± SD.

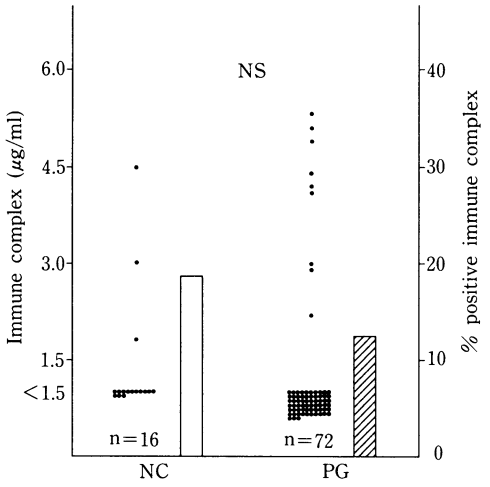

Fig. 4. Values and positive rate of immune complex in normal controls (NC) and poison gas workers (PG).

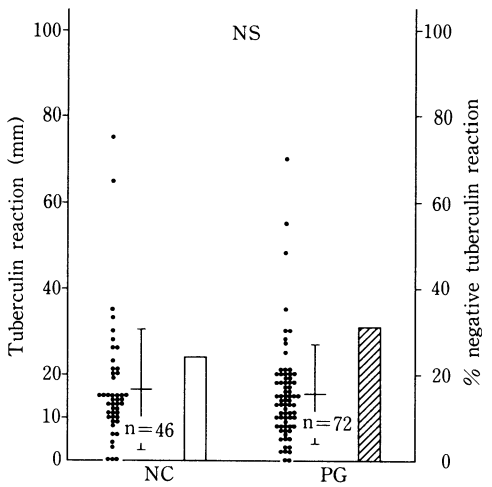

Fig. 6. Values and negative rate of tuberculin reaction in normal controls (NC) and poison gas workers (PG). Results are mean values ± SD.

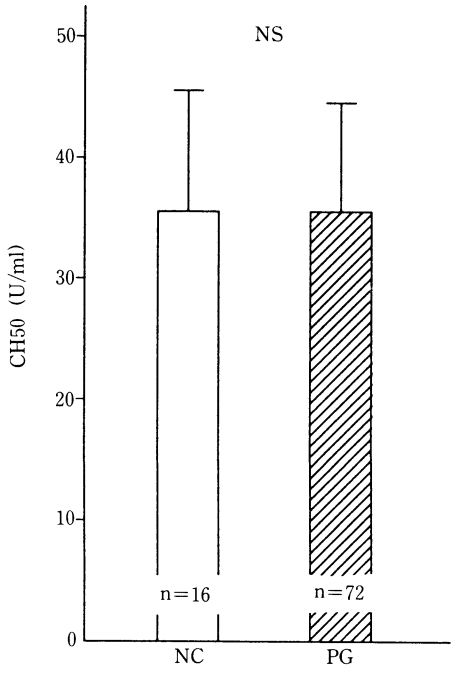

Fig. 5. Complement activity (CH_{50}) in normal controls (NC) and poison gas workers (PG). Results are mean values ± SD.

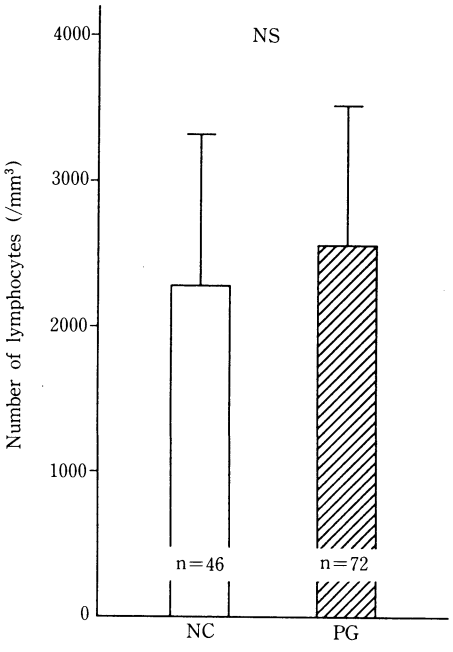

Fig. 7. Number of lymphocytes in normal controls (NC) and poison gas workers (PG). Results are mean values ± SD.

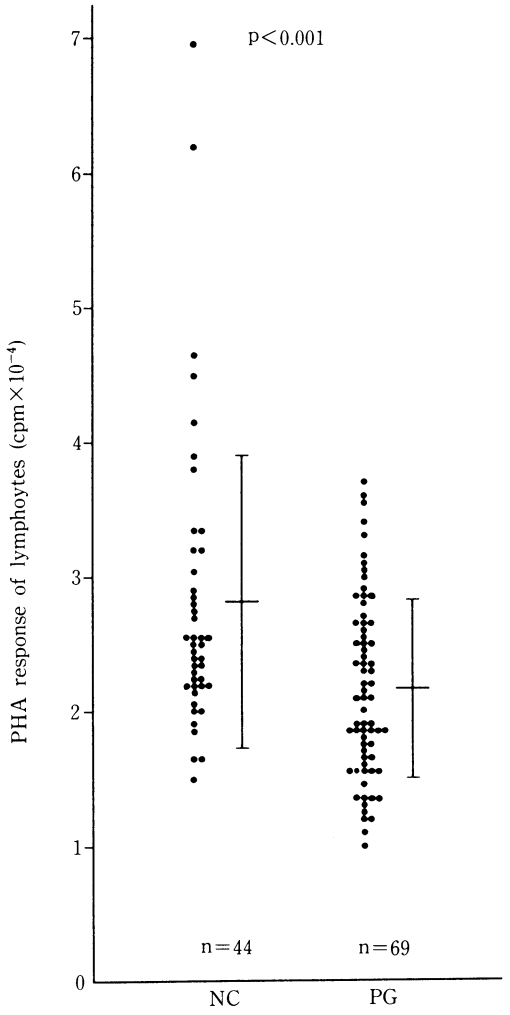

Fig. 8. PHA response of lymphocytes in normal controls (NC) and poison gas workers (PG). Results are mean values ± SD.

and one-way MLR are shown in Fig. 9. Con A response was 15,694 ± 6,694 cpm in NC and 15,587 ± 6,440 cpm in PG; PPD response was 1,465 ± 2,033 cpm in NC and 1,570 ± 1,367 in PG and one-way MLR was 29,478 ± 15,551 cpm in NC and 29,727 ± 10,877 cpm in PG with no difference observed between the two groups.

Inhibitory effects of serum on mitogenic responses and MLR in poison gas workers

As shown in Fig. 10, inhibition on PHA response was 9.1 ± 11.1% in NC and 12.0 ± 11.6% in PG; inhibition on Con A response was 11.7 ± 23.9% in NC and 15.4 ± 26.4% in PG; and inhibition on one-way MLR was 5.7±33.1% in NC and 13.0 ± 31.6% in PG. The values were generally slightly higher in PG, but the difference was not significant.

Influence of type and duration of work in the poison gas factory

As shown in Fig. 11, in the group whose duration of work was 5 years or more IgA showed a significantly higher value than those of the other two groups. Furthermore the longer the duration of work, the more significantly did the negative rate of tuberculin reaction increase. But neither influence of duration of work in other parameters nor influence of type of work in all parameters could be demonstrated.

DISCUSSION

Nishimoto et al[17,22,23] have reported that poison gas workers are a high risk group of cancer and that this is primarily attributable to the long term inhalation of minute amount of yperite gas. Carcinogenecity of yperite gas has been demonstrated in animal experiments[2,4] and it has been reported in man that in servicemen of the Allied Forces exposed to yperite gas during the first World War the incidence of respiratory tract neoplasms was high[1]. The authors therefore made a comparison of immunological parameters between poison gas workers and age matched normal controls in order to elucidate the association of carcinogenesis to immune function in poison gas workers which may be said to be a reserve group of carcinogenesis.

As for serum factors, α_1-AT, α_1-AG, α_2-HS, and C_3 were examined as so-called acute phase reactants. It is considered that α_1-AT and α_1-AG increase under tumor-bearing conditions and act immunosuppressively[11,18], while C_3 varies in autoimmune diseases[9]. Such immunoglobulins as IgG, IgA and IgM decrease in various immunodeficiencies. CEA, ferritin and β_2-M are used as tumor markers and are known to increase in various types of malignant neoplasms such as cancers of the digestive system and respiratory tract[5,7,8]. It has been reported that immune complex and complement activity show a high value in autoimmune diseases and increase in malignant neoplasms[10,19]. In contrast to the findings of the foregoing reports, poison gas workers when compared with normal controls only showed a slightly elevated

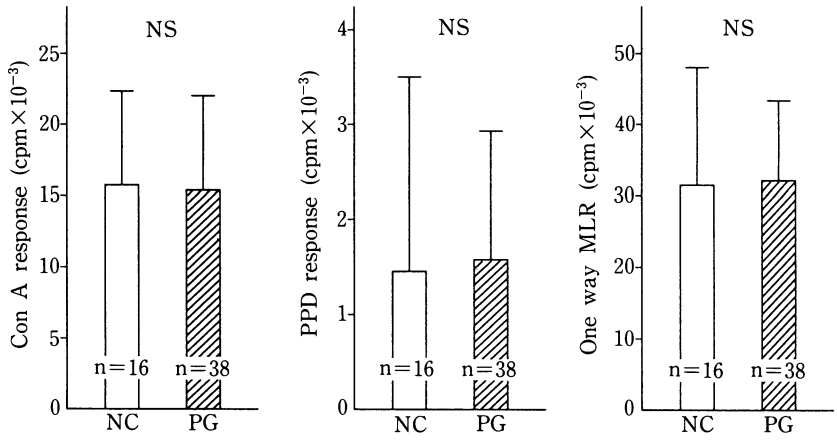

Fig. 9. Con A and PPD response of lymphocytes and one-way MLR in normal controls (NC) and poison gas workers (PG). Results are mean values ± SD.

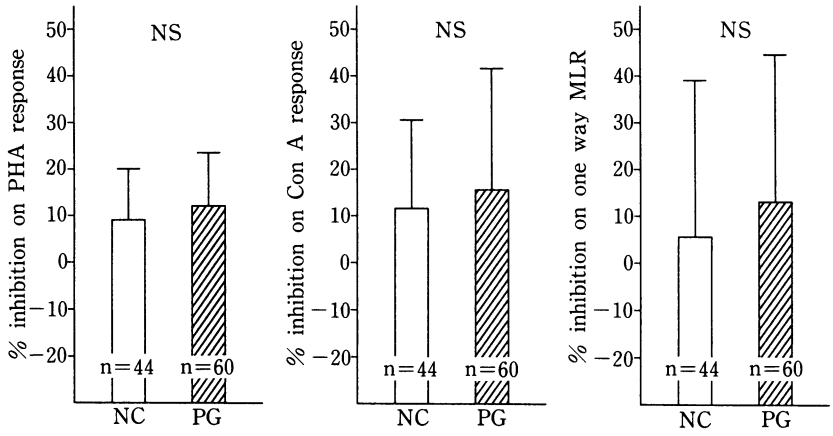

Fig. 10. Inhibitory effects of serum on mitogenic response of lymphocytes and one-way MLR in normal controls (NC) and poison gas workers (PG). Results are mean values ± SD.

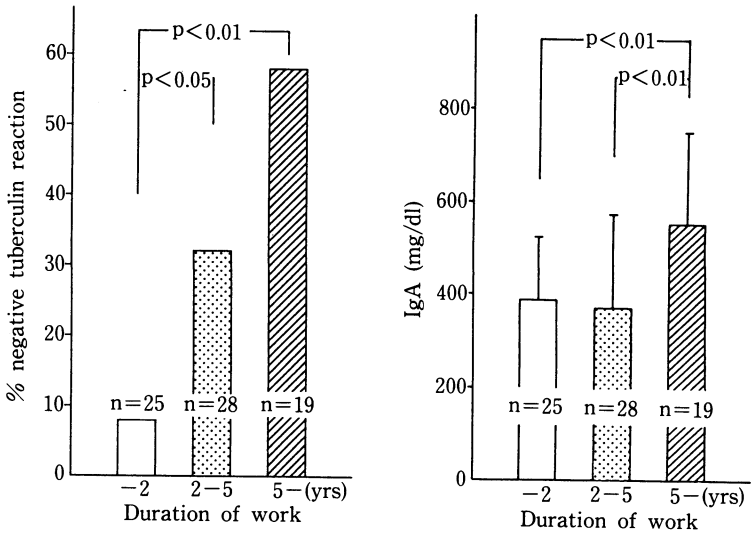

Fig. 11. Influence of duration of work in the poison gas factory on negative rate of tuberculin reaction and level of IgA. Statistical analysis are tested by means of X^2 test in negative rate of tuberculin reaction. Results are mean values ± SD in level of IgA.

value in IgA and CEA with no significant difference being demonstrable in other serum factors. In the group whose duration of work was five years or more, IgA showed a significantly elevated value. Serum IgA level is considered to be elevated in patients with chronic inflammatory diseases such as chronic bronchitis. In the group whose duration of work was five years or more, there are much more poison gas workers who seriously suffer from chronic bronchitis than the other groups. This fact seems to be the reason why serum IgA level was high in that group.

It is well known that tuberculin skin reaction, a delayed type hypersensitivity, is depressed in malignant neoplasms and it is reported to be remarkably depressed with progression of disease[6]. In contrast to this, no significant difference could be observed between poison gas workers and normal controls, but with extension of duration of work the rate of negative reaction significantly elevated.

It has been reported that the number of peripheral blood lymphocytes decreases under tumor-bearing conditions and that such decrease becomes marked in advanced cancer[24]. However, in poison gas workers in comparison with normal controls, the number of lymphocytes slightly increased. It is well known that the number of lymphocytes increases in chronic inflammatory disease. All the poison gas workers who are the subjects of the present study had moderate to severe chronic respiratory infection, chiefly chronic bronchitis, and thus it is considered that the number of lymphocytes was increased.

PHA response which is considered to be one of the T cell functions showed a significant depression in poison gas workers when compared with that of normal controls. On the other hand, it is also well known that PHA response decreases under tumor-bearing conditions and its decrease is reported to become remarkable with progression of disease[14]. In contrast to these, no significant difference could be observed in Con A and PPD response and one-way MLR in the present study.

It is also known that various immunosuppressive agents are present in the serum of cancer patients and that they act suppressively on mitogenic response of lymphocytes and MLR[20]. In contrast, the inhibitory effects of serum on mitogenic response and MLR showed generally slightly high values in poison gas workers when compared with normal controls, but significant difference could not be demonstrated. Furthermore, though not described in results, a study was made on the correlation between the foregoing serum factors and PHA response, but no significant correlation could be observed in any of the factors.

In view of the foregoing, depression in T cell function could be observed in poison gas workers which could not be attributable to serum factors and depression of functions of T cells themselves could be considered. It is considered that further detailed studies on T cell function are warranted.

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