Exp Oncol 2013
35, 3, 207–210

Experimental ONCOLOGY

ANTIBODIES AGAINST BENZO[A]PYRENE IN IMMUNIZED MOUSE AND IN LUNG CANCER PATIENTS

V.A. Ustinov[1,], V.A. Matveeva[2], M.A. Kostyanko[3], A.N. Glushkov[1]*
[1]*Institute of Human Ecology of Siberian Branch of Russian Academy of Sciences, Kemerovo 650065, Russia*
[2]*Institute of Chemical Biology and Fundamental Medicine of Siberian branch of Russian Academy of Sciences, Novosibirsk 630090, Russia*
[3]*Kemerovo State University, Kemerovo 650043, Russia*

***Aim*: To evaluate the production of antibodies against benzo[a]pyrene (BP) (Ab1) and corresponding antiidiotypic antibodies (Ab2) in mice after immunization with BP-protein conjugate and in lung cancer patients. *Materials and Methods*: The Ab1 and Ab2 levels were measured by non-competitive ELISA in blood serum of 10 mice immunized with BP-protein conjugate, and in blood serum of 288 healthy persons and 165 lung cancer patients. *Results*: The Ab1 level of was 2-fold higher than Ab2 level in blood serum of BP-immunized mice. In lung cancer patients the Ab1 level was almost 3 times higher and the Ab2 level was by 30% higher than these indexes in healthy individuals. The Ab1/Ab2 ratio was 2 in BP-immunized mice and healthy individuals and 1 in lung cancer patients. *Conclusion*: Our data have shown that the Ab1/Ab2 ratio in lung cancer patients differ from that in healthy individuals and is close to the Ab1/Ab2 ratio in BP-immunized mouse.**
***Key Words*: benzo[a]pyrene, antibodies, lung cancer.**

The immunology of chemical carcinogenesis attracted of the scientists attention for a long time [1]. In particular, it was found that animals immunization with protein conjugates of chemical carcinogens (CC) leads to raise specific antibody (Ab) against the corresponding CC in serum [2–4]. The CC-induced tumors are inhibited in immunized animals [5, 6]. The prolonged CC exposure to body develops specific immune response due to the CC interaction to proteins and DNA *in vivo*. The biotransformation enzymes are involved in that immune response [7–9]. Anti-CC are detected in the cancer patients serum [10–13].

It was supposed that in response to the "first" Ab (Ab1) against CC in the body formed the "second" antiidiotypic antibody (Ab2) [8]. The Ab1 against CC and corresponding Ab2 were observed in the serum of breast cancer patients [11].

The purpose of this paper was to figure out the Ab1 production against benzo[a]pyrene (BP) and corresponding Ab2 in mouse by immunization with BP-protein conjugate, as well as the levels of these Abs in blood serum of lung cancer patients compared to healthy persons.

MATERIALS AND METHODS

Animals. CBA mouse (10 females 3 months old) were immunized intraperitoneally with 200 mg conjugate BP with bovine serum albumin (BP-BSA) per mouse. Immunization was carried out for 4 weeks. The first immunization the antigen was injected in complete Freunds adjuvant. The incomplete Freunds adjuvant was used for the next two immunizations. Injections were performed every 2 weeks. The control group mouse (10 females 3 months old) was injected with saline on a similar basis. Blood sampling was performed from retro-orbital venous sinus of mouse prior to immunization and then once the 2nd, 4th and 6th weeks. Housing and care of the animals, as well as the experiments, corresponded to article 11 of Helsinki declaration of second Medical Association (1964), "International guidelines for conducting biomedical researches using animals" (1985) and "Rules of laboratory practice in Russian Federation" (Order of Department of Health № 267, 19.06.2003).

Clinical material. The blood serum samples of 453 people, including 165 lung cancer patients, were obtained from the Regional Clinical Oncology Center in Kemerovo (Russia). The control group included 288 healthy persons without cancer. The study was carried out with the informed consent of the patients and healthy persons.

Synthesis of conjugates. BP-BSA was synthesized by covalent coupling of hapten aldehyde group to the BSA amino groups [14].

Purification of rabbit polyclonal antibodies against BP. The anti-BP Ab was prepared from fresh serum of rabbit immunized with BP-BSA by following scheme: the first immunization was performed with antigen in complete Freunds adjuvant. The next two immunizations were in incomplete Freunds adjuvant. The affinity-purified rabbit polyclonal antibodies against BP were prepared by gamma globulin chromatography fractions using column BP-hexokinase-BrCN activated sepharose 4B [15]. After binding of gamma globulin fraction and washing of unbound proteins by sodium phosphate buffer solution (PBS), the Ab was eluted with 0.1 M glycine-HCl, pH 2.4, neutralized with 1 M Tris-HCl pH 8.0, and then were dialyzed against PBS.

ELISA. The levels of the Ab1 and Ab2 were analyzed by non-competitive ELISA [16]. The immunological polystyrene plates were coated with 50 ml of 2 mg/ml BP-BSA at 25 °C overnight (these

Received: May 25, 2013.
Correspondence: E-mail: valustinov@yahoo.com
Abbreviations used: Ab – antibody; BP – benzo[a]pyrene; CC – chemical carcinogens.

wells were called BP-BSA) or affine purified polyclonal rabbit Ab against BP at a concentration of 50 ng/ml (these wells were called AbR). Then the plates were blocked by 100 ml of PBS containing 0.5% BSA and 0.05% Tween 20 for 1 h at 37 °C with shaking. The human and mouse serum were diluted by blocking solution for the analysis at 1:100, which was half-maximal ELISA binding, at 37 °C on a shaker for 1 h. For comparison, the control wells were incubated with BSA or rabbit Ig unbounded to BP for affinity chromatography. These wells were called BSA or IgR, respectively. The Ab1/Ab2 binding to the wells was introduced into 50 ml of HRP labeled rabbit antibodies against mouse immunoglobulin G or anti-human immunoglobulin G at 37 °C on a shaker for 1 h. After each step the plates were washed 3 times with PBS/0.05% Tween 20. Absorbance was performed using tetramethylbenzidine at 450 nm. Levels of Ab1 against BP were calculated using formula: Ab1 = (OD BP-BSA) — (OD BSA)/OD BSA. The BP-BSA OD and OD BSA were optical densities with adsorbed BP-BSA and BSA. Level of antiidiotypic antibody Ab2 were calculated using Ab2 = (OD AbR) — (OD IgR)/OD IgR.

Statistical analysis. The results were analyzed with the use of Statistica 6.0 program. The data were reported as the mean ± SD. The statistical significance of differences between mean values was assessed by the Student's *t*-test. Values $p < 0.05$ were considered statistically significant. The correlation was analyzed by linear regression equation.

RESULTS

Ab against BP in the immunized mouse serum. To analyze the production of Ab1 and Ab2 against BP, we have immunized mice with BP-BSA conjugate. The Ab1 was not found in the serum of control mice injected with PBS. However, low levels of Ab2 have been found (data not shown).

Fig. 1 shows the average levels of Ab1 and Ab2 in mice of the experimental group (n = 10) immunized with BP-BSA. The levels of both Abs increased after each immunization.

The statistically significant rise of Ab1 level was observed after the second immunization with BP-BSA (compared with the first one, $p = 0.006$) and after the third one with BP-BSA (compared with the second one, $p = 0.005$). Similarly, there was a significant increase of Ab2 levels after the second and the third immunizations ($p = 0.0014$). The increase of Ab1 level was significantly higher than that of Ab2.

We calculated the Ab1 and Ab2 ratio (Ab1/Ab2) in each blood serum sample. The average values of these ratios are presented in Fig. 2.

It was found that average Ab1/Ab2 ratio was 0.5 in the initial serum samples (before immunization). So, the level of Ab1 was 2 times lower than Ab2 level in intact mice. After the first BP-BSA injection the average Ab1/Ab2 ratio increased up to 1.0 when Ab1 level reached Ab2 level. After the third BP-BSA injection the average Ab1/Ab2 ratio was significantly increased — up to 2.0. The difference between the mean Ab1/Ab2 values was statistically significant. Thus, the results presented in Fig. 1 and 2 have indicated that mice immunized with BP-BSA are characterized by significantly higher production of Ab1 than that of Ab2.

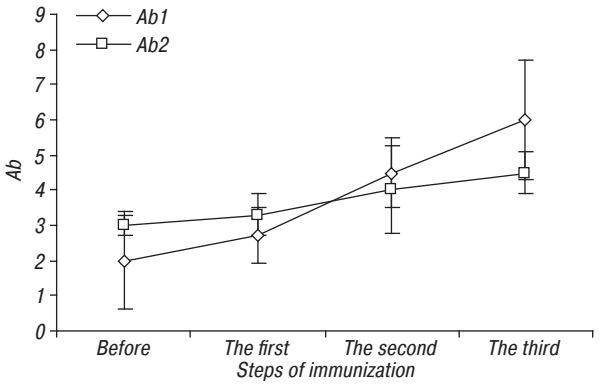

Fig. 1. The levels of Ab1 and Ab2 in blood serum of 10 mouse immunized with BP-BSA. The result of ELISA was binding immunized mouse serum to BP-BSA and to affine purified polyclonal rabbit AB against BP. The BP-BSA and Ab were adsorbed onto the polystyrene plates. The blood serum from 10 mice before immunization and after three immunizations were used. The dilution of mouse serum was 1:100, which was half of the maximum binding (IC_{50}). The level of Ab1 was determined by the formula: OD Ab1 binding to BP-BSA minus OD Ab1 binding to BSA and divided by OD Ab1 binding to BSA. The level of Ab2 was determined by the formula: OD Ab2 binding to AbR minus OD Ab2 binding to IgR and divided by OD Ab2 binding to IgR

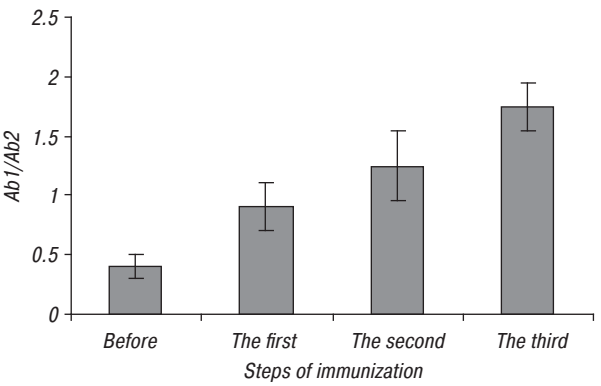

Fig. 2. The Ab1/Ab2 ratio in blood serum of mice before and after BP-BSA immunization

20 blood serum samples of the mice from experimental group after the second and third immunizations were analyzed for the Ab2 and Ab1 levels (Fig. 3). There has been observed a direct correlation between Ab1 and Ab2 levels ($r = 0.59$; $p = 0.02$; $y = 0.4x + 0.1$, where y is Ab2, x is Ab1). Linear regression analysis has shown an increase of Ab1 level by 1.0 when the Ab2 level of increased only by 0.4.

The relations between Ab2 and Ab1 levels in blood serum before immunization and after the first injection of BP-BSA were not revealed.

The AB against BP in blood serum of lung cancer patients. We have analyzed Ab1 and Ab2 levels in blood serum samples of healthy persons (n = 288) and lung cancer patients (n = 165) by ELISA using the BP-BSA and affine purified BP polyclonal rabbit AB immobilized on 96 wells plates to determine of Ab1 and Ab2, respectively (Fig. 4).

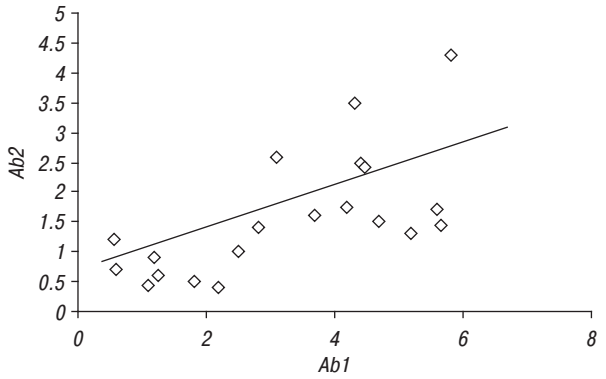

Fig. 3. The dependence of Ab2 level from Ab1 level in blood serum after the second and third immunizations (n = 20)

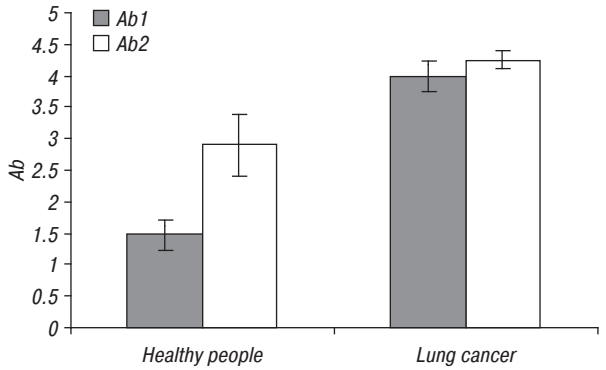

Fig. 4. The levels of Ab1 and Ab2 in healthy individuals (n = 288) and in lung cancer patients (n = 165). The experiments design was similar to the same mouse serum experiments.

The level of Ab2 was 2 times higher than Ab1 level (p = 0.0015) in blood serum of healthy people. In lung cancer patients, the Ab1 level was 3 times higher than in healthy individuals (p = 0.001), and the level of Ab2 was 30% higher than in healthy persons (p = 0.0023).

Fig. 5 shows the Ab1/Ab2 ratio in blood serum of healthy persons and lung cancer patients which in lung cancer patients was almost 2 times higher than in healthy persons (0.39 ± 0.1 and 0.95 ± 0.12, respectively). Interestingly, in the healthy persons Ab1/Ab2 ratio was close to that value in non-immunized mice, while in lung cancer patients it tended to reach Ab1/Ab2 ratio in immunized mice, but was twice lower because of different Ab2 levels.

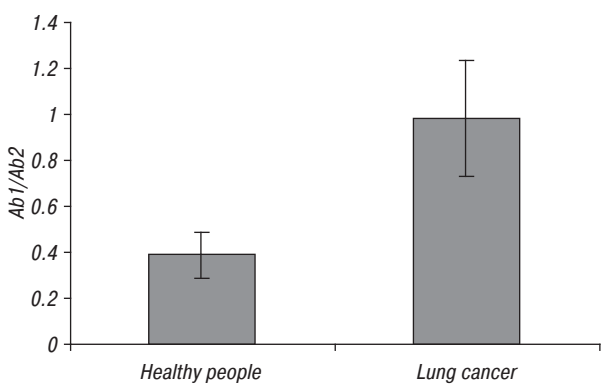

Fig. 5. The Ab1/Ab2 ratio in healthy individuals and lung cancer patients

There were determined the direct correlations between Ab2 (y) and Ab1 (x) in healthy persons (r = 0.5; p = 0.0015) and lung cancer patients (r = 0.6; p = 0.001) (Fig.6 *a* and *b*, respectively).

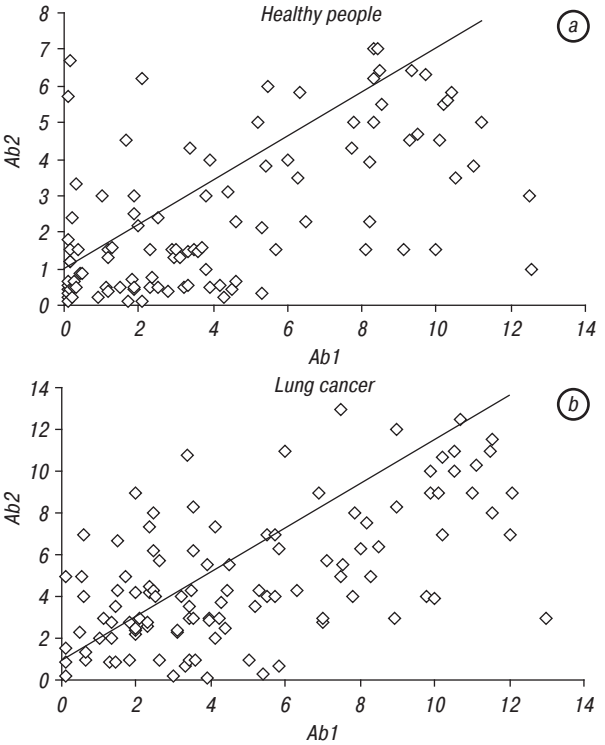

Fig. 6. The correlations between levels of Ab1 and Ab2 in blood serum of healthy individuals (*a*) and lung cancer patients (*b*)

The relationships were described by the linear regression y = 0.5x + 0.11 for healthy persons and y = 0.9x + 0.05 for lung cancer patients. This means that in healthy persons Ab1 level increased by one and Ab2 level increased by 0.5 what was similar to data obtained in mice after immunization with BP-BSA (y = 0.4x + 0.1). The Ab1 level increased by one and the Ab2 level increased by 0.9 in lung cancer patients.

Cigarette smoking has been demonstrated in a number of epidemiological and clinical studies as the major factor that contributes to the development of lung cancer [17]. We have grouped the blood serum samples of lung cancer patients and blood serum samples of healthy persons into the groups of smokers and nonsmokers (healthy individuals: smokers n = 93 *versus* nonsmokers n = 195; lung cancer patients: smokers n = 129 *versus* nonsmokers n = 36), but did not reveal significant differences in Ab1 and Ab2 levels between these subgroups.

DISCUSSION

In present research, the Ab1 and the corresponding Ab2 levels were checked in BP-BSA immunized mouse, in healthy individuals, and lung cancer patients.

In mice the Ab1 were produced after BP-BSA immunization, but were not detected in the blood serum before such immunization and in control mice injected with PBS. The significant Ab1 rise was detected after the third BP-BSA immunization. The Ab2 production occurred in response to increased Ab1 synthesis caused by intensive immunization, but it did not reach Ab1 level. The correlation between Ab1 and Ab2 lev-

els in immunized mouse was moderate — r = 0.6 for mice, r = 0.59 for healthy persons, and r = 0.5 and lung cancer patients.

As it follows from our experimental and clinical data, the production of Ab1 and Ab2 against BP in non-immunized mice was similar to those in healthy persons: an average Ab2 level was higher than average Ab1 level, and Ab1/Ab2 ratios were also close to each other. The Ab1 and Ab2 production against BP in immunized mice possesses similar patterns to that in lung cancer patients: average levels of Ab1 and Ab2 were increased, while average Ab1/Ab2 ratio were 2 and 1 for immunized mouse and lung cancer patients, correspondingly.

We could speculate about high levels of Ab1 and Ab2 in blood serum of lung cancer patients compared with healthy persons. Probably, the concentration of genotoxic BP metabolites in lung cancer patients is higher than these in healthy persons [5–8] leading to increased Ab1 production. The high level of Ab1 leads to the production of Ab2 which could bind and block Ab1, resulting in decreased immunological BP resistance and hypothetically — increased risk of cancer.

The relationship between cancer and cigarette smoking has been well established in many types of cancers [17]. In our study, no difference was detected in the groups of smokers and non-smokers, possibly, due to small number of examined samples. The influence of individual factors including industrial and domestic factors on the levels and ratio of Ab1 and Ab2 would be the next subject of the research. Also, it would be interesting to figure out if Ab1/Ab2 ratio depends on the activity of CC metabolizing enzymes.

ACKNOWLEDGEMENTS

This study was supported by the RFFI grant number 10–04–98003 r_sibir_a — Regional Competition SIBERIA. We especially thank Sherina E.A and Gurov E.A. for fruitful help in ELISA. Also we would like to thank Titov V.A for the samples of lung cancer patients blood and healthy people blood.

REFERENCES

1. Vermeersch KA, Styczynski MP. Applications of metabolomics in cancer research. J Carcinogenesis 2013; **12**: 9 (doi: 10.4103/1477–3163.113622).

2. Creech H, Oginsky E, Tryon M. Immunological studies of hydrocarbon-protein conjugate. Cancer Res 1947; **7**: 301–4.

3. Cernohorská H, Klimešová S, Lepša L, ***et al.*** Influence of immunization with non-genotoxic PAH-KLH conjugates on the resistance of organisms exposed to benzo(a)pyrene. Mutat Res 2012; **18**; **742**: 2–10.

4. Glushkov A, Klimov I, Kostyanko M, ***et al***. Preparation of antibodies against fluoromethylbenzanthracene. Exp Oncol 1994; **16**: 437–40 (in Russian).

5. Peck R, Peck E. Inhibition of chemically induced neoplasia by immunization with an antigenic carcinogen-protein conjugate. Cancer Res 1971; **31**: 1550–4.

6. Wanczyk H, Barker T, Rood D, ***et al***. Cloning and characterization of a hybridoma secreting a 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-specific monoclonal antibody and recombinant F(ab). Toxins 2013; **5**: 568–89.

7. Andric NL, Kostic TS, Zoric SN, ***et al.*** Effect of a PCB-based transformer oil on testicular steroidogenesis and xenobiotic-metabolizing enzymes. Reprod Toxicol 2006; **22**: 102–10.

8. Pauk N, Klimesova S, Kara J, e***t al.*** The relevance of monitoring of antibodies against the polycyclic aromatic hydrocarbon (PAH) and PAH-DNA adducts in serum in relation to lung cancer and chronic obstructive pulmonary disease (COPD). Neoplasma 2013; **60**: 182–7.

9. Chagnaud JL, Amara A, Faiderbe S, ***et al***. Identification of a benzo[a]pyrene-like binding protein involved in circulating immune complexes of patients with mammary tumors. Cancer Lett 1995; **91**: 253–6.

10. Glushkov A, Klimov I, Anosova T, ***et al***. Antibodies to polycyclic aromatic hydrocarbons in sera of patients with malignant mammary tumors. Exp Oncol 1996; **18**: 284–6 (in Russian).

11. Glushkov A, Anosova T, Nebesnaya N, ***et al***. Isotipical special features of antibodies to polycyclic aromatic hydrocarbons in patients with cancer of mammary gland, stomach, colon, and rectum. Exp Oncol 1996; **18**: 426–8 (in Russian).

12. Glushkov A, Anosova T, Anosov M, ***et al.*** Are there any interrelations between immunogenic activity of chemical carcinogens and tumor antigens in cancer patients. Rus J Immunol 1997; **2**: 50–4 (in Russian).

13. Kostyanko MV, Glushkov AN. Synthesis of gaptenprotein conjugate. Patent 16.02.1998. RF 2141114.

14. Glushkov AN, Kostyanko MV, Cherno SV, ***et al***. Immunoassay of antibodies to benzo[a]pyrene. Exp Oncol 1998; **20**: 74–6 (in Russian).

15. Glushkov A, Krasilnikova K, Polenok E, ***et al***. Antibodies to benzo[a]pyrene, estradiol, and progesterone and polymorphisms CYP1A2*1F, GSTT1, and GSTM1 in pregnant women with congenital malformation of the fetus. Rus J Immunol 2012; **2**: 162–9.

16. Center MM, Ferlay J, Ward E, ***et al***. Global cancer statistics. CA Cancer J Clin 2011; **61**: 69–90.