

ANTISERA TO POLYCYCLIC AROMATIC HYDROCARBONS AND THEIR APPLICATION ON THE DETECTION OF CHEMICAL CARCINOGENS IN BLOOD SERA OF ONCOLOGICAL PATIENTS

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Aim: To investigate the cross-reactivity of the hyperimmune antisera of animals (rabbits) and the sera of oncological patients to polycyclic aromatic hydrocarbons (PAHs) of a similar chemical structure. **Methods:** Reactions of antibodies with haptens have been estimated by non-competitive and competitive ELISA with synthesized protein conjugates of PAHs as a coating antigen. **Results:** All the model rabbit antisera have been stated to react with anthracene, chrysene, pyrene, benzo(a)pyrene and benz(a)anthracene irrespective of the hapten used to immunize animals. The cross-reactivity of serum antibodies to all five PAHs has been found in blood samples of oncological patients. **Conclusion:** It is recommended to use the conjugates of anthracene, chrysene and pyrene for detecting human antibodies to more dangerous environmental carcinogens — benzo(a)pyrene and benz(a)anthracene.

Key Words: cross-reactivity, antibodies, polycyclic aromatic hydrocarbons (PAHs), protein conjugates.

Earlier we have reported a new method of the synthesis of polycyclic aromatic hydrocarbon–protein conjugates and their application on analysis of antibodies (AB) to benzo(a)pyrene in human blood sera [4, 5]. Cross reactions of hyperimmune sera of animals as well as blood sera of oncological patients with different PAH have been studied. This article summarizes the results of these investigations.

MATERIALS AND METHODS

Synthesis of PAH — protein conjugates. The conjugates of benzo(a)pyrene (Bp), benz(a)anthracene (Ba), chrysene (Cr), anthracene (Ac) and pyrene (P) with bovine serum albumin (BSA) and yeast hexokinase (HK) were synthesized by the method described earlier [4]. To study the influence of chemical structure and lengths of the spacer group on the interaction of AB with hapten, it was necessary to synthesize 6 pyrene conjugates with BSA (P2–P7). They were synthesized from N-(1-pyrenylmethyl)-3-alanine (P2), 1-glutamimidopyrene (P3), 1-succinamidopyrene (P4), 1-pyrene-carboxaldehyde-O-(carboximethyl)oxime (P5), 4-oxo-1-pyrenebutyric acid (P6) and 1-pyrenebutyric acid (P7) by the technique given below.

From a solution of 0.1 g BSA in 1 ml 0.1 M of sodium hydroxide and 9 ml acetone the protein was precipitated with 0.1 ml of acetic acid, and the precipitate was thrice washed with 10 ml of acetone and thrice — with 5 ml of anhydrous N,N-dimethylformamide (DMF), 0.1 ml N-methylmorpholine being added into DMF during the

third washing. The precipitate was placed into a vessel with a magnetic stirrer, then we added 1.5 ml of DMF and activated hapten prepared at –5 °C from 0.05 mM pyrene with a spacer group, 10 µl N-methylmorpholine and 5 µl isobutyl chloroformate (10 µl for P2) in 1 ml DMF into the vessel. The mixture was stirred for 5 h, left overnight, and diluted with 10 ml of acetone with 0.1 ml of the acetic acid. The precipitate was centrifuged and then washed 4 times with 5 ml of 40% DMF solution in acetone, twice — with 5 ml of 20% DMF solution in acetone and thrice — with 10 ml of acetone. The precipitate was immediately dried up under vacuum.

The method allowed us to synthesize the conjugates containing 15 molecules of pyrene per a molecule of BSA. The method turned out to be highly efficient due to the absence of water in the reaction medium resulting in 80% of mixed anhydride having taken part in the reaction.

The chemical structure of all types of conjugates is presented on Fig. 1. Conjugates Bp, Ba, Ac and Cr were linked with protein by the –CH₂–NH– spacer similarly to conjugate P1, presented in Fig. 1.

Animal immunization. Rabbits were immunized according to the following scheme: PAH — BSA conjugates (2 mg) were given intramuscularly every week, the 1st injection in 0.5 ml of complete Freund's adjuvant (Sigma, USA), the 2nd injection — in 0.5 ml of incomplete Freund's adjuvant, the 3rd injection — in 1 ml of distilled water. Then every 2 weeks booster injections of 1 mg of conjugate in 1 ml of distilled water were administered. Blood was taken in a two-month period after the beginning of immunization once every two weeks.

Analysis of anti-PAH-antibodies in blood sera. The properties of the obtained conjugates and AB were investigated by ELISA as described earlier [4]. The rabbit hyperimmune antiserum was diluted, placed into wells coated with PAH–HK conjugates. AB to haptens were determined using peroxidase-labelled goat anti-

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Abbreviations used: Ac — anthracene; AB — antibody; Ba — benz(a)anthracene; Bp — benzo(a)pyrene; BSA — bovine serum albumin; Cr — chrysene; DMF — N,N-dimethylformamide; DNA — deoxyribonucleic acid; HK — yeast hexokinase; PAH — polycyclic aromatic hydrocarbon; P — pyrene.

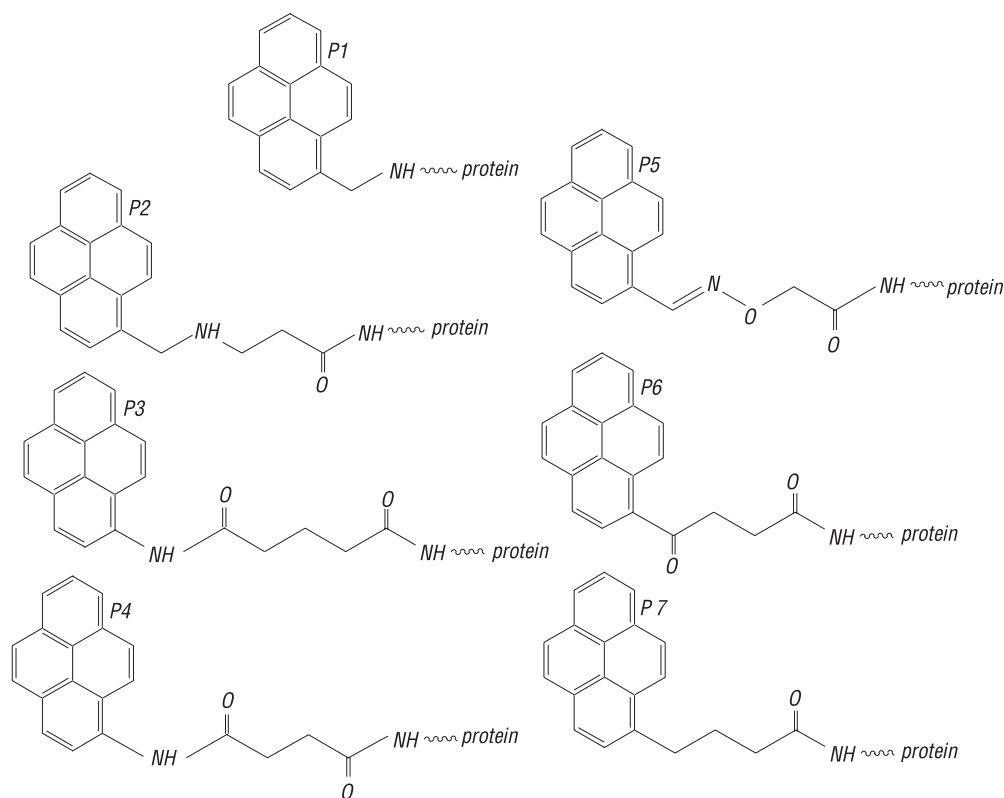


Fig. 1. Chemical structure of PAH — protein conjugates

rabbit IgG AB (kindly gifted by dr. P.P. Laktionov, Novosibirsk Institute of Biological Chemistry and Fundamental Medicine, Russia). The use of BSA as a protein-carrier for immunization and HK for immunoassay (because of their structural differences) enables us to determine AB only to haptens. In this case we found the maximum dilution of antiserum (1 : 4000–1 : 16000, depending on the sample used) at which immunoassay is adequate. Then, using competitive ELISA, hyperimmune antisera in dilutions 1 : 4000–1 : 16000 were incubated with conjugates of PAH–protein (in different concentrations) for 1 h at 37 °C before placing into wells. The subsequent stages of the immunoassay were carried out using the technique mentioned in [4].

64 samples of blood serum of patients with various malignant tumors were investigated. Each sample of blood serum was diluted (dilution 1 : 100, 1 : 200, 1 : 400 and 1 : 800 in PBS with 0.5% BSA) to avoid the cross-reaction with the protein-carrier in immobilized PAH — BSA conjugates. The wells of microtiter plates were coated with PAH — BSA conjugates with Bp, Ba, Ac, Cr and P. As the control, the wells with immobilized BSA without haptens were used. AB were defined using peroxidase-labelled goat anti-human IgA AB. AB of this isotype were found earlier in the oncological patients [1, 4]. AB were considered as positive samples if their optical density exceeded at least 2-fold that of the “control”.

RESULTS AND DISCUSSION

The ability of hyperimmune antisera to link with immobilized PAH — HK conjugates was investigated in model experiments. Any sample of antisera obtained after immunization of the rabbits with BSA with Bp, Ba,

Ac, Cr or P conjugates was found to contain AB cross-reacting with each hapten, the binding with hapten used for immunization being sometimes least effective. As a rule, the greatest bond was observed for immobilized Ba, irrespective of the hapten used to immunize animals. The antiserum obtained after immunization with the Bp–BSA conjugate which reacted with all the haptens in the similar way was the only exception.

The results of ELISA of antisera after the rabbits were immunized with conjugate P1, are presented in Fig. 2. As seen, the bond of AB with HK (control) is absent. The minimum reaction was observed with Ac. In this case the AB titre did not reach 1 : 32000. The maximum bond is found for Ba. Its optical density remained still very high even for antiserum in 1 : 64000 dilution. The binding curves for P, Bp and Cr occupy an intermediate position.

The ability of PAH — HK conjugates to compete with similar immobilized conjugates for binding with AB was

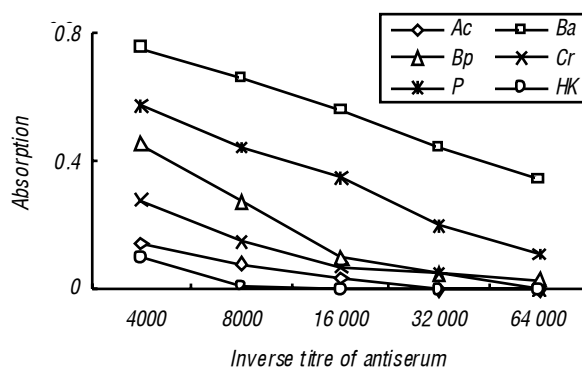


Fig. 2. ELISA of rabbit antiserum immunized with pyrene — BSA conjugate (P1)

investigated. Ba was found to be the greatest competitor for any antiserum. The results of the analysis of the rabbit antiserum immunized with P1 conjugate are submitted in Fig. 3. As shown, a 50% decrease in the signal is observed for Ba — HK with the concentration 0.1 $\mu\text{g/ml}$ in antiserum. At the same time, 50% decrease in the signal is observed for Ac — HK, Bp — HK, Cr — HK and P — HK is observed in the 1–10 $\mu\text{g/ml}$ range. Therefore, the affinity of the AB to Ba appeared to be, at least, one order of magnitude more than the one for the other PAH, in spite of the usage of the conjugate P1 as a immunogene. Although, the conjugates with the same spacer-group ($-\text{CH}_2-\text{NH}-$) were used in these experiments, the observed regularities may be supposed to be due to the structure of haptene rather than the spacer group.

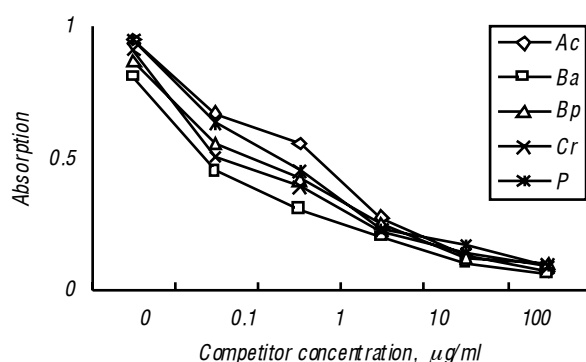


Fig. 3. Competitive ELISA of rabbit antiserum immunized with pyrene — BSA conjugate (P1) with certain PAH

We tried to optimize the method of immunoassay of AB to PAH by modifying the chemical structure and the length of the spacers in PAH–protein conjugates. For this purpose 6 types of pyrene conjugates with BSA were synthesized (see Fig. 1). The rabbit antisera after the immunization with the conjugate P1 were tested in noncompetitive and competitive immunoassays. In the former case, the antisera reacted only with the immobilized P1 but not with P2–P7 conjugates. A similar result was achieved for the latter (Fig. 4). The comparison of the chemical structures of P1 and P2 conjugates shows that the increase of a spacer length does not increase the binding of hapten with AB. Also, the replacement of the ($-\text{CH}_2-\text{NH}-$) group in the conjugate P1 by any other group in conjugates P3–P7 results in the decrease of the efficiency of their binding with AB.

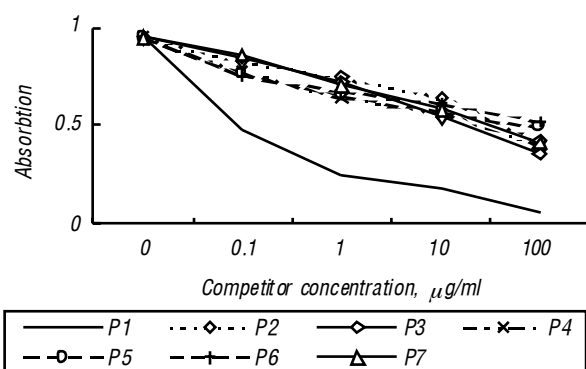


Fig. 4. Competitive ELISA of rabbit antiserum immunized with pyrene — BSA conjugate (P1) by pyrene with different spacers (P2–P7)

To investigate the cross-reactivity of AB to a particular PAH conjugate of different haptens with the same spacer group must be used, otherwise the modification of the spacer might result in the change of the AB affinity to a particular PAH.

Thus, the model experiments have shown that during the immunization of animals by any of PAH–protein conjugates the AB to a wide PAH range are formed. In their environment humans undergo the influence of many compounds from this chemical class. They are metabolized by mixed–function cytochrome–dependent oxygenases that may form active metabolites which form adducts binding covalently with DNA and proteins, i.e. they become haptens [6, 8]. The presence of AB to DNA adducts with metabolites Bp, Ba, Cr in healthy people was reported in [6–8]. The authors [6–8] do not consider AB to react with PAH as they usually do with haptens. They assume AB to react with structurally modified DNA sites. Besides, the ability of AB in a sample of serum to react with either one of the used adducts or with 2–3 adducts simultaneously was revealed.

The usage of protein conjugates with Bp [1] or with fluorinated Ba [3] which is a synthetic molecule allowed us to reveal the AB which may react with PAH molecules as with haptens. Therefore, it is obvious that AB reacting with it are formed during contacts of humans with environmental PAH.

To obtain additional information about the mechanism of the formation and functions of AB to chemical carcinogens in humans and especially to estimate their importance as a prognosis biomarker of individual carcinogenic risk it is necessary to use the methods of analysis meeting the following requirements: 1) at the 1st stage of widescale pre–screening, positive samples of sera should be selected excluding the appearance of erroneously negative samples with low costs and maximum safety for a researcher; 2) at the 2nd stage of detailed studying the AB (+) samples the isotype of AB, the range of specificity (which PAH is involved in a particular human) and the affinity should be determined.

We investigated the cross–reactions of blood sera in oncological patients with Bp, Ba, Cr, Ac and P.

Table shows the quantify of AB–positive samples when the serum reacted with one (or more) of the five used PAH. It is seen that 83% of AB samples are positive at 1 : 100 and 1 : 200 dilutions. Further dilution results in the decrease of the percentage of AB (+) samples. Therefore, the optimum serum dilution at the first stage is 1 : 100.

Besides, it is seen that 31–56% of samples reacted with the used PAH. At the same time 2–11% reacted only with one of the 5 PAH. Thus, single specificity occurs much less frequently than polyspecificity. The binding with one Bp was recorded only in one case out of 64. Since Bp is considered as a possible carcinogen for humans [2], it is important to know whether it can be changed by others, less dangerous for a researcher and more informative haptens for immunoassay of AB.

We investigated how frequently the positive serum reaction on Bp ($n = 20$) coincides with the positive reactions on other PAH. Such coincidences were found

Table. The amount of AB (+) sera reacting with PAH in oncological patients

	Serum dilution							
	1 : 100		1 : 200		1 : 400		1 : 800	
	n	%	n	%	n	%	n	%
AB(+)	53	83	53	83	45	70	42	66
Ac (+)	36	56	35	55	24	38	21	33
Ba (+)	28	44	31	48	24	38	20	31
Bp (+)	20	31	15	23	15	23	13	21
Cr (+)	36	56	32	50	24	38	14	22
P (+)	35	55	33	52	24	38	22	34

The note: The total number of samples n = 64

for P in 19 cases (95% out of n = 20), for Cr — in 18 (90%), for Ac — in 17 (85%) and for Ba — in 16 (80%). To reveal AB to BP, it is possible to use less dangerous P with the probable coincidence 95%.

Ba is also considered as a probable carcinogen for humans [2]. Similar coincidences were found for Ba (n = 28): for Ac — 23 (82%), for Cr — 22 (79%), for P — 19 (68%) and for Bp — 16 (57%); i.e. to reveal AB to Ba it is possible to use less dangerous Ac with the probable coincidence 82%.

Thus, the combination of Ac and P as haptens allows one to find AB reacting with Ac and P in addition to AB to Bp (coincidence 95%) and AB to Ba (coincidence 82%), the coincidence being 83% from the total amount of AB (+) samples (44 cases from 53).

If the combination Ac and Cr is used, the efficiency of detection of AB to PAH rises slightly. The probability of Bp coincidence is 95%, Ba — 90%, the total amount of AB (+) — 87% (46 cases from 53).

In order to detect all AB (+) cases it is necessary to use the combination of four haptens: Ac, Cr, P and Ba. Thus the application of more dangerous PAH — Bp is excluded but 100% detection of all AB (+) is achieved.

Apparently, at the first stage of screening during the investigation of formation mechanisms and functions of AB to PAH it is sufficiently enough to use the combination of two haptens — Ac and Cr. However, the screening aimed at detecting the individual carcinogenic risk must be carried out with a wider range of haptens, i.e. Ac, Cr, P and Ba, excluding Bp.

In conclusion, the experimental data on the interaction between PAH and modelled rabbit AB to PAH

are given. The models used by the present authors allow one to detect a subtle specificity of AB to low weight compounds with a very similar chemical structure. It is obvious that AB in polyclonal hyperimmune sera are capable of reacting with any PAH, whichever PAH – protein immunogene is used.

To detect AB to PAH in blood sera in humans during screening it is expedient to use Ac, Cr and P as haptens, excluding carcinogenic Bp and Ba.

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АНТИСЫВОРОТКИ К ПОЛИЦИКЛИЧЕСКИМ АРОМАТИЧЕСКИМ УГЛЕВОДОРОДАМ И ИХ ИСПОЛЬЗОВАНИЕ ДЛЯ ВЫЯВЛЕНИЯ ХИМИЧЕСКИХ КАНЦЕРОГЕНОВ В СЫВОРОТКАХ ОНКОЛОГИЧЕСКИХ БОЛЬНЫХ

Цель: исследование перекрестных реакций гипериммунных антисывороток животных (кролей) и сывороток больных онкологического профиля к близким по химической структуре полициклическим ароматическим углеводородам (ПАУ). **Методы:** реакции антител с гаптенами оценивали с помощью неконкурентного и конкурентного ELISA, где в качестве покрывающего антигена использовали синтезированные белковые конъюгаты ПАУ. **Результаты:** все полученные модельные антисыворотки кролей перекрестно реагировали с антраценом, бенз(а)антраценом, бензо(а)пиреном, пиреном и хризеном независимо от того, каким именно антигеном были иммунизированы животные. **Способность к перекрестному реагированию** обладали сыворотки крови больных онкологического профиля. **Заключение:** рекомендуется использовать конъюгаты антрацена, хризена и пирена для выявления антител человека к более опасным канцерогенам — бензо(а)пирену и бенз(а)антрацену.

Ключевые слова: иммуноанализ, антитела, полициклические ароматические углеводороды (ПАУ).