ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 5, pp. 569–574. © Pleiades Publishing, Ltd., 2009.
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EXPERIMENTAL ARTICLES

The Effect of Alkylhydroxybenzenes on the Antigen-Binding Capacity of Antibodies

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Received August 19, 2008

Abstract—The effect of the chemical analogues of microbial extracellular autoregulators belonging to alkylhydroxybenzenes (AHB), hexylresorcinol (HR), and methylresorcinol (MR), on the interactions between specific antibodies and the corresponding antigens was studied. Nonlinear dependency of the inhibition of binding of AHB-modified antibodies on the AHB chemical structures and concentrations was revealed by enzyme immunoassay. Hexylresorcinol was shown to decrease the antibody affinity and avidity indices and simultaneously increase the indices of nonspecific binding of AHB-modified antibodies to antigens, thereby promoting the formation of "false" antigen–antibody complexes. The nonspecificity of the influence of AHB on the antigen-binding capacity of antibodies is an important characteristic of these effects, which allows us to consider AHB as unique "superhaptenes".

Key words: antibodies, avidity, binding specificity, antibody modification, alkylhydroxybenzenes, microbial adaptogens.

DOI: 10.1134/S0026261709050063

Alkylhydroxybenzenes (AHB) are a broad group of bioactive molecules of microbial and plant origin [1]. For example, in bacteria of the genera *Azotobacter, Pseudomonas, Micrococcus, Thioalkalivibrio*, etc., these compounds act as extracellular autoregulators, adaptogens capable of inducing a transition of bacterial cells into a hypometabolic and anabiotic (dormant) state [2–5].

The known mechanisms of the effects of AHB are based on their capacity for a number of physicochemical interactions (hydrophobic, electrostatic, and hydrogen bonds) with membrane lipids [6], proteins [7, 8], and nucleic acids [9], which may result in an alteration of the structural organization and functional activity of subcellular structures and biopolymers. The universality of the effect of AHB as chemical chaperones [2, 7] promotes the development of the effects in heterologous systems. For example, at micromolar concentrations of AHB homologues differing in the lengths of their alkyl radicals, changes in the catalytic activity of some enzyme proteins of microbial, plant, and animal origin were demonstrated [2, 7, 9, 10], as well as a decrease in receptor affinity to fibrinogen at the platelet surface [11].

The goal of the present work was to study the effects exerted by the chemical analogues of microbial AHB (HR and MR), differing in the lengths of their alkyl radicals, on antibodies (nonenzymatic human and animal immunoproteins) when determining their main functional characteristic, the capacity for specific interactions with the relevant antigens.

MATERIALS AND METHODS

Methylresorcinol (MR, molar mass = 124) and hexylresorcinol (HR, molar mass = 194) (99.9% purity level) (Sigma, United States) were used as the chemical analogues of microbial alkylhydroxybenzenes. Water solutions of these compounds at working concentrations of 5×10^{-6}, 1.25×10^{-5}, 2.5×10^{-5}, 5×10^{-5}, 1.25×10^{-4}, 2.5×10^{-4}, and 5×10^{-4} M were used to modify the antibodies. Maturation of the modified structure of the immunoprotein was attained by preincubation of the antibodies–AHB mixtures at 37°C for 60 min prior to each experiment. In the control variants, intact antibodies were mixed with distilled water and incubated under the same conditions.

The main experiments exploring the effect of AHB on the antigen-binding capacity of the antibodies were carried out using the VektoTokso-IgG Kits (Vektor-Best, Novosibirsk, Russia) for quantitative enzyme immunoassay of immunoglobulins G specific for *Toxoplasma gondii*. The control specimens from the kits with known activities measured in international units (ME/ml) were used as antibody sources. In a separate series of experiments, the VektoRubella-IgG and VektoRubella-IgM test systems were used in order to assay

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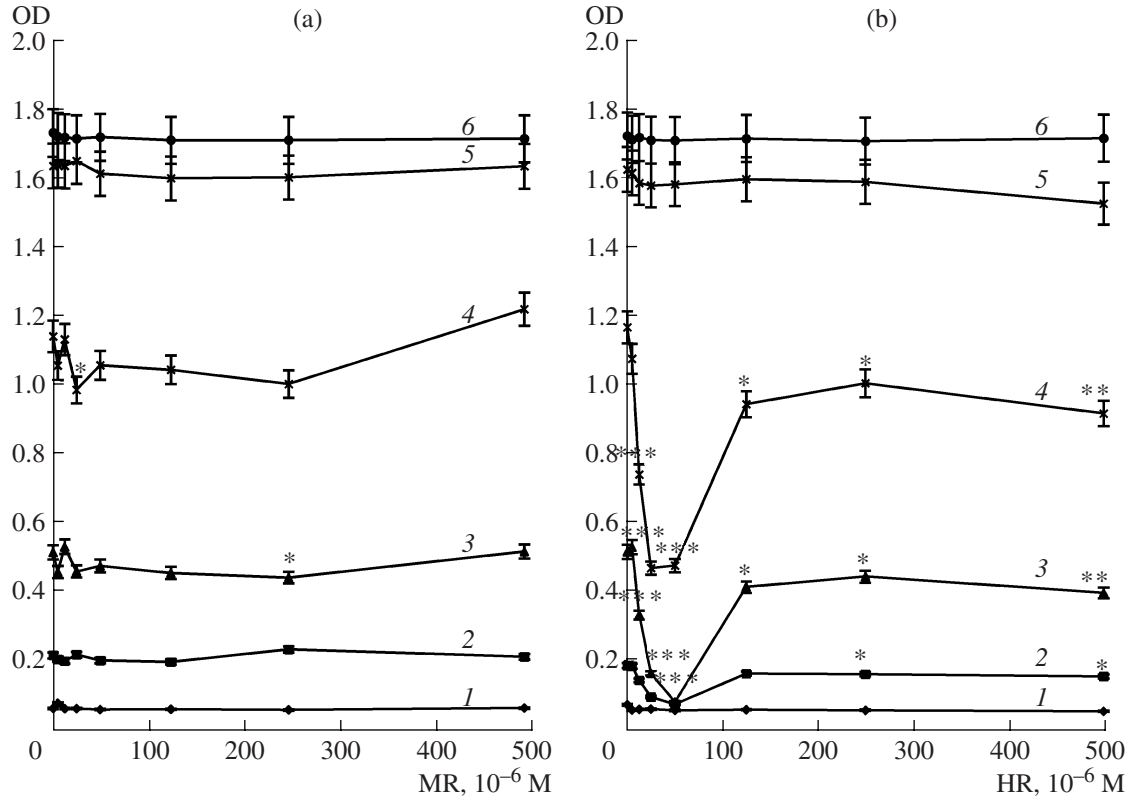

Fig. 1. The binding intensities of MR- (a) and HR-modified (b) antibodies against *Toxoplasma gondii*: the abscissa shows the AHB concentration, 10^{-6} M; the ordinate shows the optical density (OD) values, relative units; the antibodies were used in the following concentrations (ME/ml): 0 (*1*); 5 (*2*); 12.5 (*3*); 25 (*4*); 50 (*5*); and 100 (*6*). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

the relative binding indices of the relevant positive and negative specimens of the control sera against the *Rubella virus.*

The effect of AHB on the indices of antibody affinity was studied by quenching tryptophan fluorescence [12] on a Fluorat-02 Panorama spectrofluorimeter (NPF Lumex, Russia). In this case, we used a commercial preparation of normal human immunoglobulins (6.14×10^{-9} M) as antibodies. The immunoglobulin fluorescence was excited at 300 nm and measured at 336–340 nm. The fluorescence was quenched with the nitroaromatic ligand 2,4-dinitrophenol (Aldrich, Germany) added to the antibodies as 20-μl aliquots of 1×10^{-6} M solution until a molar excess of haptene was achieved.

The effect of AHB on the antibody avidity was studied by denaturing enzyme immunoassay using the VektoRubella-IgG-avidity test system as an example. Urea (8 M) was used as a denaturing agent. The avidity index (AI) was calculated using the formula:

$$\mathrm{AI} = \frac{\mathrm{OD}_{\mathrm{denat}}\mathrm{EIA}}{\mathrm{OD}_{\mathrm{direct}}\mathrm{EIA}} \times 100\%,$$

where $\mathrm{OD}_{\mathrm{direct}}$ and $\mathrm{OD}_{\mathrm{denat}}$ are the antibody binding indices before and after treatment with the denaturing agent, respectively.

The experiments were performed in at least five replicates. The results were statistically treated using the SPSS software package.

RESULTS AND DISCUSSION

The effects of antibody modification by alkylhydroxybenzenes. The *T. gondii*-targeted antibodies were preincubated in the presence of an AHB homologue, and their indices of binding to the corresponding antigens sorbed in the wells of polystyrene plates were then determined. A number of effects depending both on the structure of the used AHB homologues and the concentrations of the interacting components were observed (Fig. 1). For example, methylresorcinol (Fig. 1a), within the whole range of concentrations used, had virtually no effect on the binding of the MR-modified antibodies to antigens: significant small-scale changes were observed only in two cases, i.e., at the MR concentrations of 2.5×10^{-4} and 2.5×10^{-5} M and the antibody concentrations of 12.5 and 25 ME/ml (Fig. 1a, lines *3* and *4*); the binding indices decreased to 85.57 ± 3.92 and $86.36 \pm 3.54\%$, respectively, as compared to the control values ($P < 0.05$).

Unlike methylresorcinol, utilization of high hexylresorcinol concentrations in a range of 5×10^{-6}–5×10^{-5} M

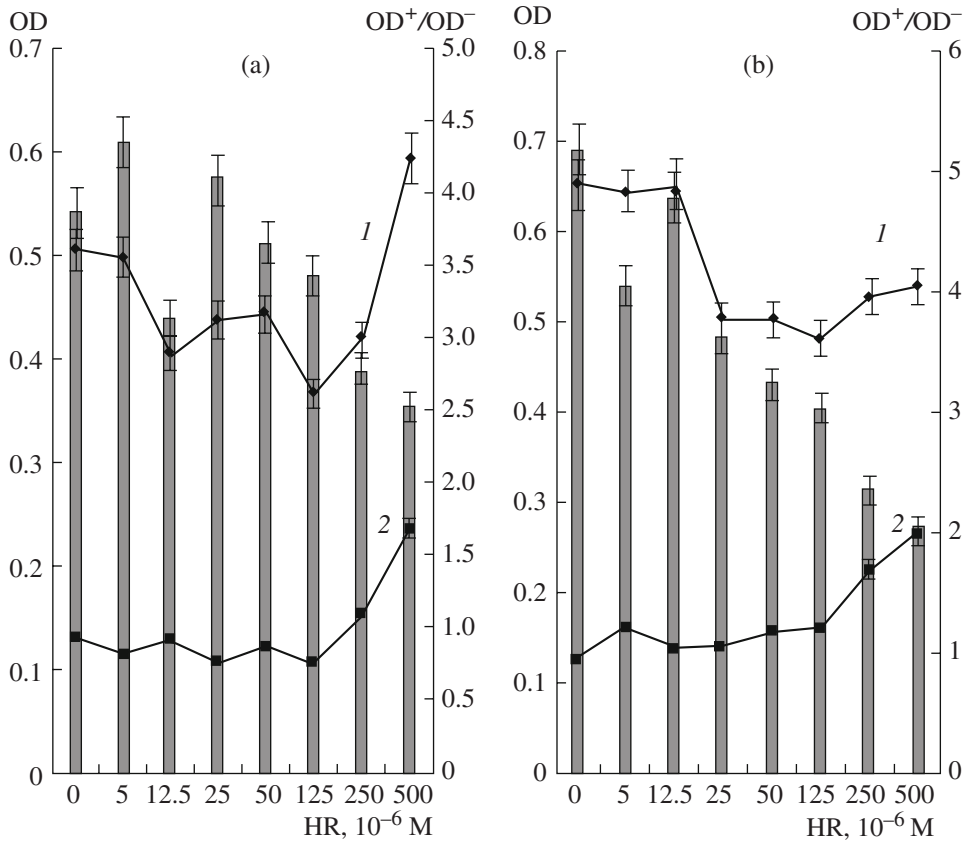

Fig. 2. The binding indices of HR-modified G (a) and M (b) immunoglobulins specific for *R. virus*: the abscissa shows the HR concentration, 10^{-6} M; the ordinate (left) shows the optical density (OD) values, relative units; the ordinate (right) shows the ratios between the binding indices of the positive and negative sera OD^+/OD^-. Designations: *1*, OD values for the positive sera; *2*, OD values for the negative sera; the bars demonstrate the ratios between the binding indices of the positive and negative sera(OD^+/OD^-).

(Fig. 1b) resulted in a much more pronounced inhibition of the formation of antigen–antibody complexes. The maximum effect was observed at the HR and antibody concentrations of 5×10^{-6} M and 12.5 ME/ml, respectively (Fig. 1b, line *3*), when the binding index was $14.86 \pm 0.58\%$ of the control values ($P < 0.001$); in the variant with the antibody concentration of 25 ME/ml, the effect was pronounced (Fig. 1b, line *4*). However, a further increase in the HR concentration did not result in a proportional increase in the effect; on the contrary, it partially restored the binding index.

An essential trait of these effects was that the degree of their intensity varied depending on the antibody concentrations. The most pronounced effect of hexylresorcinol was observed in the case of its incubation in the presence of antibodies in a concentration of 5–25 ME/ml, when the antigen-binding indices were 15–40% of the control value obtained at HR concentration from 2.5×10^{-6} to 5×10^{-5} M. Thus, the significant role of the antibody : AHB molar ratio in inhibition of the antigen-binding capacity of the antibodies was demonstrated.

On the whole, the results obtained demonstrate the possibility of direct interaction of a hydrophobic AHB homologue with the sites located in the active antibody center. This results in disruption of the specific binding of antibodies with the corresponding antigens. The chemical composition of AHB and the effects caused by these compounds allow us to consider them as haptens (low-molecular-weight compounds which are able to specifically inhibit the formation of antigen–antibody complexes) [13]. Significant differences exist, however, between the effect of AHB and those of the "classical" haptens. These differences consist both in the nonlinear concentration dependencies of the effects caused by AHB and in the nonspecific effect of AHB on antibodies.

The latter was confirmed by the results of our experiments on the effect of AHB on the antigen-binding capacity of the antibodies specific to other antigens, namely, to those of the *R. virus*. During this experiment, all the above-described effects of AHB on *T. gondii* were reproduced. For instance, methylresorcinol had virtually no influence on the studied properties of the G and M antibodies specific for *R. virus* (data not presented), whereas an increase in the HR concentration up to 1.25×10^{-4} M resulted in a progressive decrease in their antigen-binding capacity (Fig. 2). The maximum effect was accompanied by the binding indices of

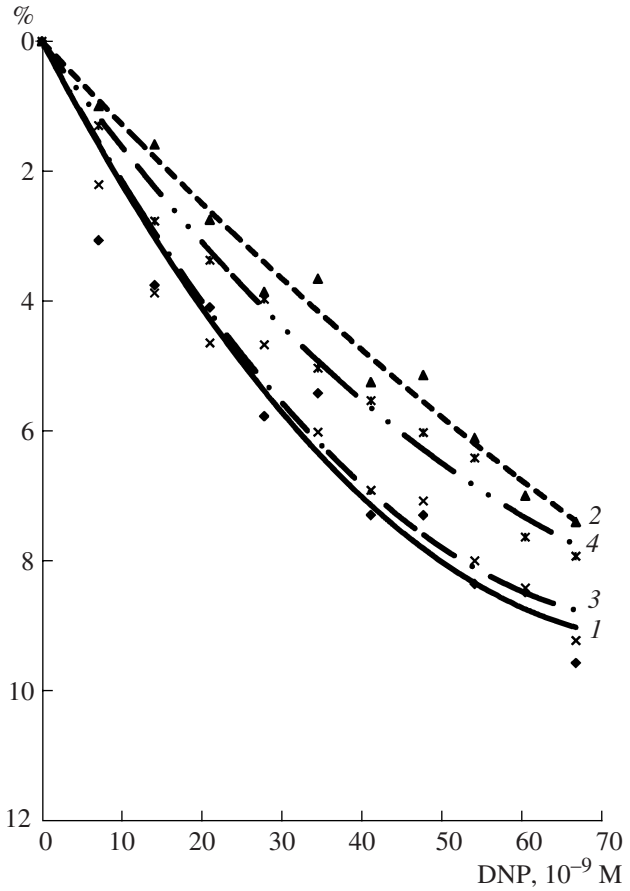

Fig. 3. Changes in the quenching rate of tryptophan fluorescence of antibodies by hapten at various HR concentrations: the abscissa shows the hapten (DNP) concentration, 10^{-9} M; the ordinate shows the degree of fluorescence quenching; HR concentrations (M): 0 (*1*); 5×10^{-6} (*2*); 5×10^{-5} (*3*); 5×10^{-4} (*4*).

72.02 ± 3.60% and 73.58 ± 3.94% of the control values ($P < 0.01$) when immunoglobulins G and M, respectively, were used as target objects (Figs. 2a and 2b). As in the experiments with the antibodies against the *T. gondii* antigens, a further increase in AHB concentrations partially restored the binding indices, which possibly has its own explanation.

When analyzing these results, it should be taken into account that, in our experiments with the VektoRubella-IgG and VektoRubella-IgM test systems for enzyme immunoassay, both "positive" and "negative" (not containing the antibodies against the *R. virus*) control sera were used for calculating the threshold values of specific binding. While the above-described effects of AHB were revealed for "positive" sera (Fig. 2, line *1*), a paradoxical two-fold increase in the binding indices of "negative" sera was observed (Fig. 2, line *2*) in the presence of high HR concentrations. This may be considered as a partial change in the ability of the nonspecific antibodies of the "negative" serum to bind with *R. virus* antigens due to modification of their structure upon interaction with HR. The partial restoration of the antigen-binding capacity of HR-modified antibodies of the "positive" sera registered in this range of HR concentrations may therefore also be a result of unspecific binding.

The above suggestions were confirmed by the results of an additional series of experiments aimed at investigation of the nonspecific binding of antibodies against thyreoperoxidase (TPO) (in a concentration of 0–1500 ME/ml) to *T. gondii* antigens. Modification of antibodies against TPO with high MP (and, especially, HR) concentrations was demonstrated to result in an increase in the binding indices by 24.59 ± 1.25% of the control values. An altered degree of hydrophobicity of the protein molecules resulting from their interaction with AHB is among the possible explanations of the formation of "false" antigen–antibody complexes; as was previously demonstrated for the enzymatic protein lysozyme [14], this may result in a similar alteration of its substrate specificity.

Thus, the application of the VectoRubella test system and the calculation of the ratios of binding to the "positive" and "negative" sera (OD^+/OD^-) enabled us both to confirm the two-phase character of AHB effect on formation of the antigen–antibody complexes depending on the concentration of these factors and to state an increased unspecific binding of AHB-modified antibodies together with suppression of their specific binding.

The effect of alkylhydroxybenzenes on the specific properties of the antibodies. To study the effects of AHB interacting with immunoproteins in detail, the effect of hexylresorcinol was determined on the affinity and avidity of the antibodies, a measure of the quality and quantity of the intensity of their binding with the corresponding antigens.

To study the affinity which is the qualitative indicator of the binding intensity of a given active center of an antibody molecule with the corresponding antigenic determinant, quenching of the tryptophan fluorescence of the antibodies (normal human immunoglobulins) by the nitroaromatic ligand 2,4-dinitrophenol (DNP) was applied.

In the control variants, successive saturation of the solution of normal human immunoglobulins with a hapten resulted in the progressive decrease in the fluorescence rates (Fig. 3), typical of low-affinity antibodies and defined by Q_{max} = 38.54%. In this case, the dependency of the fluorescence rates (FlR) on the DNP concentration was described by the exponential equation $\mathrm{FlR} = e^{\left(\frac{\mathrm{DNP} + 980 \pm 79}{717 \pm 56}\right)}$.

In the experimental variants, preincubation of the antibodies with hexylresorcinol had a weak effect on the fluorescence intensity, which may be attributed to the residual fluorescence of hexylresorcinol in the spectral range used. At the same time, preincubation of the

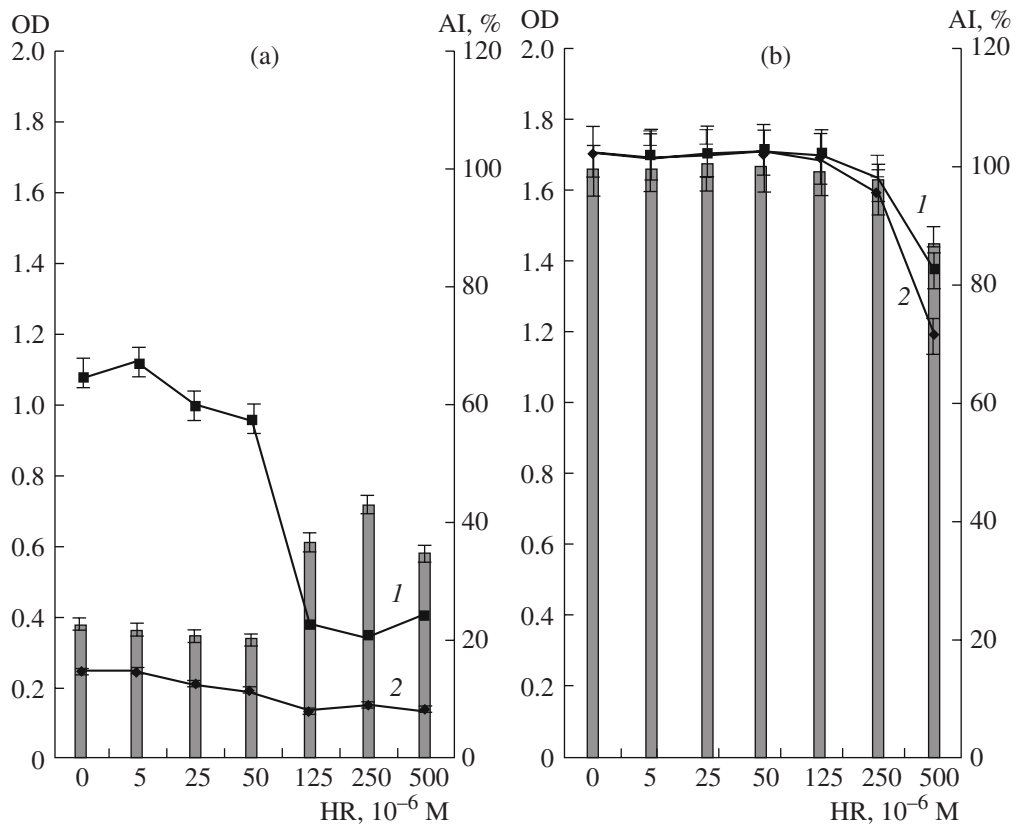

Fig. 4. Changes in the binding and avidity characteristics of low- (a) and high-avidity (b) antibodies at various HR concentrations: the abscissa shows the HR concentration, 10^{-6} M; the ordinate (left) shows the optical density (OD) values, relative units; the ordinate (right) shows the antibody avidity index (AI), %; *1*, binding capacity of the antibodies before treatment with the denaturing agent; *2*, binding capacity of the antibodies after treatment with the denaturing agent; the bars demonstrate the values of antibody avidity.

antibodies in the presence of AHB affected their capacity for the subsequent interaction with DNP (Fig. 3). A decrease in Q_i (quenching of fluorescence after supplementing the solution with each hapten dose) was the main result, accompanied by an increase in the values of the normalizing factors in the above-mentioned equation. In this case, all the parameters indicated a steady tendency towards a decrease in the affinity of HR-modified antibodies.

This conclusion was confirmed and additional effects were observed when studying the avidity of the AHB-modified antibodies, which may serve as a quantitative indicator of the ability of the whole protein molecule to bind with a polydeterminant antigen. In these experiments, two serum specimens that contained low- and high-avidity antibodies against the *R. virus* (Figs. 4a and 4b) with the initial values of the avidity index (AI) of 22.58 ± 0.97% and 99.49 ± 3.48%, respectively, were studied by denaturing enzyme immunoassay.

Preincubation of low-avidity antibodies with HR initially resulted in a pronounced inhibition of their binding with the antigens which was most pronounced in the concentration range from 5×10^{-5} to 5×10^{-4} M (Fig. 4a). In turn, the treatment of the obtained complexes with a denaturing agent resulted in an "apparent" increase in the avidity index up to AI = 34.42 ± 1.55% ($P < 0.01$). This situation may be due to the fact that HR-modified antibodies retained their capacity for interaction with the relevant antigens only at the initially relatively high binding capacity, while the least avid antibodies lost their capacity for binding prior to treatment with the denaturing agent. The fact that the relative amount of antibodies that were preincubated in the presence of hexylresorcinol and retained their binding to respective antigens after denaturation did not increase and, on the contrary, comprised of about a half of unmodified antibodies provides a strong argument in favor of this suggestion.

At the same time, preincubation of high-avidity antibodies in the presence of hexylresorcinol had a weak effect on the indices of direct binding with antigens. On the other hand, the registered avidity indices showed a tendency towards a decrease only at HR concentration of 5×10^{-4} M. In this case, a decrease in the AI value to 86.15 ± 3.52% ($P < 0.05$) was observed (Fig. 4b).

Thus, the effect of AHB on the ability of AHB-modified antibodies to bind to the polydeterminant antigen depends on the initial avidity indices of immunÓproteins and was most pronounced in the case of

low-avidity antibodies. Analysis of the results obtained allows us to conclude that AHB, especially hexylresorcinol, are capable of interacting with antibodies, thereby blocking the production of antigen–antibody complexes. However, unlike "classical" haptens with similar properties, AHB exhibit the following behavior patterns: (1) they are capable of nonspecific blocking of the antigen-binding capacity of antibodies; (2) they can partially change their specificity; and (3) they exhibit a nonlinear dose–effect dependency between the modifying AHB concentration and the level of inhibition of the antigen-binding capacity of the antibodies. These facts allow us to consider the studied small AHB molecules as a kind of "superhaptens".

In addition, the results obtained allow us to make a suggestion about the possible mechanism of the above-described effects of AHB. It seems likely that the major contribution to modification of the protein globules is from the interactions between the hydrophobic alkyl radicals of hexylresorcinol and the hydrophobic amino acids in the active center of the antibody. The hydroxyl radicals of the aromatic ring of hexylresorcinol may have an additional effect on the hydration rate of the protein molecule. As a result, the protein conformation changes to the optimal one for binding the specific antigen determinant, simultaneously with the emergence of the partial affinity to nonspecific determinants. As the AHB concentrations increase, alkylhydroxybenzenes begin to interact with the hydrophobic sites within the whole biopolymer globule, which may result in its stabilization and the partial restoration of the binding indices due to nonspecific interactions of immunoproteins with the antigen.

Our further investigations will be aimed at the more detailed study of these phenomena, as well as of the effects exerted by AHB on the stability of various antibodies under various denaturing conditions.

ACKNOWLEDGMENS

This work was supported by the Russian Foundation for Basic Research, project no. 08-04-99078-r-ofi.

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