role in this situation is evidently played by the general increase in the intensity of metabolism of rodents in spring and fall. Data showing that activity of adaptive reactions of rodents is weaker in the spring and fall [1] are in good agreement with the powerful development of catalepsy in these seasons [1].

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IMMUNOLOGIC AND PHARMACOLOGICAL ACTIVITY OF ATROPINE–PROTEIN CONJUGATES

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KEY WORDS: atropine; conjugates; immune response; antibody formation.

Antibodies specific for low-molecular-weight pharmacologically active compounds are widely used in practical and experimental medicine and, in particular, in pharmacology. To obtain specific antibodies, animals are immunized with artificial conjugated antigens (conjugates), in which low-molecular-weight pharmacologically active substances play the role of hapten determinants of immunochemical specificity. Protein conjugates of atropine have previously been used to obtain antibodies specific for atropine, and to develop a method of quantitative radioimmunoassay of the cholinolytic in biological fluids [9, 13]. However, the activity of these conjugates as inducers of a humoral immune response specific for atropine, and also the time course of specific antibody formation to these antigens have not been studied. There is no information in the literature on pharmacologic activity of atropine conjugates.

The aim of this investigation was to study immunologic (induction of atropine-specific antibody formation) and pharmacological (cholinolytic) activity of atropine conjugates when injected into experimental animals, within a wide dose range.

EXPERIMENTAL MEHOD

Experiments were carried out on 500 non-inbred and inbred (CBA) mice weighing 16-20 g and on 20 rabbits, of different sexes, weighing 2-3 kg. The atropine conjugates were synthesized under conditions similar to those described previously: p-carboxyphenylazoatropine–bovine serum albumin (PCPAA-BSA) by the method in [13], atropine hemisuccinate–BSA (AHS-BSA) by the method in [9]. The number of hapten groups on the carrier (epitopic density) was determined spectrophotometrically. The PCPAA-BSA conjugate has 10, the AHS-BSA conjugate had 6 epitopes of chemically modified atropine. For immunization of the mice and rabbits, the conjugates were injected mixed with Freund's complete adjuvant, in doses of 0.5 to 10 mg/kg, subcutaneously: Mice received two injections at an interval of 7 days; rabbits

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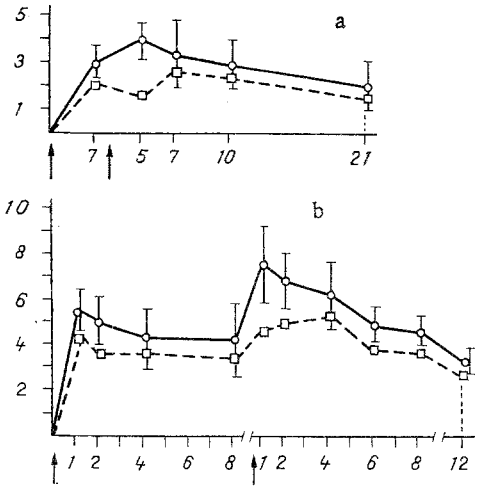


Fig. 1

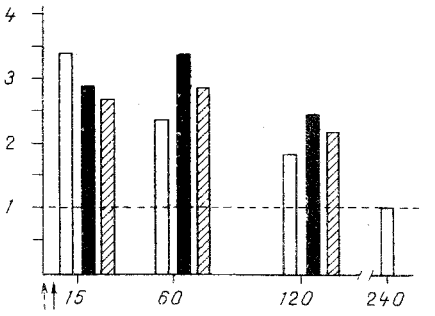


Fig. 2

Fig. 1. Time course of humoral immune response to atropine in mice (a) and rabbits (b) immunized with protein conjugates in a dose of 5 mg/kg. Abscissa, time after injection of conjugates: a) days, b) weeks; ordinate, serum antibody titer, $\log_2 N$, where N denotes the titer of sera in IHAT. Values of titer given with confidence interval m+mt, at the P = 0.05 level of significance. Continuous line denotes titer of atropine-specific antibodies; broken line, titer of ME-resistant antibodies (IgG).

Fig. 2. Comparative activity of atropine sulfate (5mg/kg) and protein conjugates of atropine (50 mg/kg) in carbachol antagonism test *in vivo*. Abscissa, time (in min) after injection of atropine sulfate (broken arrow) or of atropine conjugates (continuous arrow); ordinate, value of protective index. Unshaded columns, protective index for atropine sulfate; black columns, for AHS-BSA conjugates; obliquely shaded columns, for PCPAA-BSA conjugate.

received repeated injections, in cycles of three injections with an interval of 30-50 days between cycles. The cholinolytic activity of the atropine conjugates was tested in experiments on non-inbred mice. The atropine-like activity of the conjugates was estimated in the immunized animals 1 and 24 h after injection of the antigens in doses of 1 and 10 mg/kg and at the peak of antibody production (5th day after reimmunization).

In another series of experiments the atropine-like activity of the conjugates was determined in mice receiving a single subcutaneous injection of the antigens in doses of 1, 10, 25, and 50 mg/kg without adjuvant. In this case cholinolytic activity of the atropine conjugates was estimated 15, 60, and 120 min after injection. The atropine-like activity of the conjugates was compared with the pharmacological effect of atropine sulfate, injected subcutaneously in a single dose of 5 mg/kg body weight. To investigate the central cholinolytic effects of the conjugates and of atropine a model of arecoline-induced tremor was used [2]. To assess the peripheral cholinolytic effect of the conjugates and atropine, the protective action of the conjugates and atropine against acute carbachol poisoning was studied. Carbachol was injected intramuscularly in a single dose of between LD_0 and LD_{100} for mice in five or six groups (six animals in each group, with a dose step of 0.25-1 mg/kg. LD_{50} of carbachol was calculated in each experimental group by the method in [1]. The effectiveness of the cholinolytic action of the conjugates and atropine was estimated from the shift of LD_{50}, by calculating the protective index. Intact animals (control 1) and mice immunized with BSA (control 2) were used as the controls.

The time course of atropine-specific antibody formation was studied by determining the serologic characteristics of sera of immunized animals. The sera were obtained by the usual method and complement was inactivated by heating the sera to 56°C for 30 min. Titers of atropine-specific antibodies were determined by the indirect hemagglutination test (IHAT) in a micromodification [10], using erythrocytic diagnostic sera with hapten groups of chemically modified atropine, prepared as described by the writers previously [7]. The relative serum

concentration of IgG-antibodies was estimated by treating the sera with 0.2 M solution of 2-mercaptoethanol (ME) at 37°C for 30 min, after which the IHAT was performed. The content of IgG-antibodies (ME-resistant immunoglobulins) in the sera was expressed by the ratio

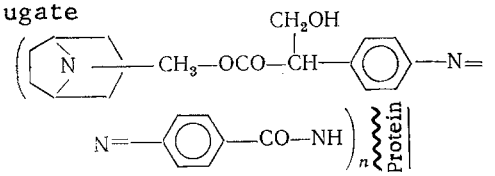
$$\frac{\text{titer of serum after treatment with ME}}{\text{titer of untreated serum}} \times 100\%.$$

To determine the comparative intensity of antibody formation to the hapten, the titer of serum antibodies to the protein carrier also was determined, by carrying out the IHAT with BSA-sensitized erythrocytic diagnostic serum.

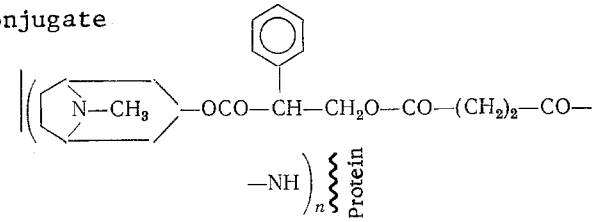
EXPERIMENTAL RESULTS

Determination of the toxicity of the atropine conjugates showed that in accordance with Sanotskii's classification [8], protein conjugates of atropine are virtually nontoxic compounds: In experiments on mice and rabbits none of the experimental animals died after receiving an injection of the conjugates in doses up to 100 mg/kg. Both types of conjugates, whose structural formulas are as follows:

PCPAA-BSA conjugate



AHS-BSA conjugate



were found to be active inducers of atropine-specific antibody formation. Consequently, covalent binding of atropine through the chemical groups of its molecule used for this purpose leaves the immunologically important functional groups free. Maximal formation of antibodies with specificity for the hapten determinant of chemically modified atropine took place during immunization of the experimental animals with atropine conjugates in doses of 5 to 10 mg/kg. After injection of the conjugates in lower or higher doses the intensity of the humoral immune response specific for atropine decreased. Data on the time course of the humoral immune response, obtained with mice and rabbits after injection of atropine conjugates into them in the optimal dose, are given in Fig. 1.

It will be clear from Fig. 1 that after reimmunization marked activition of the humoral immune response of secondary type was observed. Subsequent antibody production was long-lasting and was accompanied by a change of class of the circulating immunoglobulins. Whereas in the early stages after reimmunization IgM predominated in the sera of the experimental animals, later antibodies belonging to the IgG class predominated in the sera. The change of classes of immunoglobulins circulating in the serum, under these circumstances, was accompanied by an increase in their affinity for atropine. Thus the time course of the humoral immune response to the hapten determinant of chemically modified atropine corresponds to the process described for other haptens, and usually defined as "maturation of the immune response" [11, 12]. The humoral response to atropine is comparable in intensity with the immune response to conjugates of morphine, barbiturates, serotonin, and other low-molecular-weight biologically active compounds. It will also be evident that, in the doses used for immunization, atropine covalently bound with a high-molecular-weight protein carrier does not exhibit the immune depressive properties which have been described for cholinolytics [3-6]. Otherwise, it would be impossible for a full humoral immune response to atropine to be realized. The important point is that, in doses optimal for induction of the humoral immune response (1-10 mg/kg), atropine conjugates do not possess cholinolytic activity. Neither central nor peripheral pharmacological effects

similar to the cholinolytic effects of atropine could be recorded 1-24 h after injection of the conjugates, or during the period of maximal production of serum antibodies to the hapten (5th-7th days after reimmunization). The situation described above changed with an increase in the doses of conjugates which were injected into the experimental animals. For instance, after injection of atropine conjugates of both types into mice in a dose of 50 mg/kg which, even if given repeatedly, does not induce an immune response with active formation of circulating serum antibodies specific for atropine, atropine-like cholinolytic effects of the conjugates were recorded. Under these circumstances, cholinolytic activity of prophylactically injected conjugates in the arecoline tremor model was not present, but when the protective effectiveness of the conjugates was determined against acute carbachol poisoning in mice, a protective action of prophylactic injections of the conjugates was observed, and was comparable with the effect of atropine (Fig. 2).

The experimental results are evidence that protein conjugates of atropine, depending on the dose used, possess different kinds of biological activity. In doses of 1 to 10 mg/kg atropine conjugates of both types induced a homoral response of secondary type on repeated injection, with immunologic memory formation and with active production of serum antibodies with specificity for atropine. In doses exceeding 25 mg/kg, protein conjugates of atropine, when given in a single injection, exhibit peripheral cholinolytic activity. Consequently, when atropine is present as a component of conjugates, despite its covalent binding with the high-molecular-weight carrier, it does not lose its pharmacological activity characteristic of the free ligand. Manifestations of this activity are accompanied by reduced ability of the conjugates to induce a humoral immune response specific for atropine. The central cholinolytic effects of atropine are absent, for the high molecular weight of the conjugates (over 70,000 daltons) prevents them from passing through the blood–brain barrier, whereas the peripheral muscarinic cholinergic target structures are accessible for the high-molecular-weight (polymeric) form of the cholinolytic, based on albumin.

It can thus be concluded from the experimental data as a whole that immunologic and pharmacological reactive structures and mechanisms may be simultaneously involved in the realization of the response of the body to xenobiotics of this kind. The technical approach used, implying the obtaining of conjugates of pharmacologically active haptens in which the original ligand is bound with a high-molecular-weight carrier through various chemical groupings, may prove useful for studying the immunologic and pharmacological functional significance of different chemical groups of low-molecular-weight biologically active compounds.

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