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RESEARCH ARTICLE

Study of the specifics of the formation of immunoglobulin G and immunoglobulin M antibodies to low molecular weight substances (synthetic cannabinoids and antidepressants) in laboratory mice

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ABSTRACT

Background: To date, there is little evidence in the literature about the effects of synthetic cannabinoids "spice" and antidepressants on the mammalian immune system. **Aims and Objectives:** The study of the formation of immunoglobulin (Ig)G and IgM class antibodies to synthetic cannabinoids "spice" and antidepressants for a comparative assessment of the role of the class of antibodies in the detection of dependence on psychotropic drugs. **Materials and Methods:** Conjugates were obtained with four antidepressants: Faverin, carbamazepine, sertraline, and fluoxetine and two synthetic cannabinoids of the "spice" type – AB-FUBINACA and ADB-CHMINACA in conjunction with the carrier protein-bovine serum albumin. The serological activity of synthesized conjugates based on the protein matrix was determined. **Results:** Data were obtained on the determination of antibodies to the studied synthetic cannabinoids and antidepressants by enzyme-linked immunosorbent assay, and the immunoreactivity of mouse antibodies against heterologous haptens was established. **Conclusion:** The article examines in detail the issues related to the comparative analysis of the formation of IgG and IgM antibodies to synthetic cannabinoids and antidepressants in mice in the experiment. It has been established that IgM antibodies play a significant role in the diagnosis of psychotropic substances.

KEY WORDS: Antibodies; Specific Immunoglobulin; Spice; Opiates; Barbiturates; Cannabinoids; L-Adrenaline; Dopamine Hydrochloride; Enzyme-linked Immunosorbent Assay; Haptens

INTRODUCTION

Studying the immune response when using psychotropic substances (PS) and determining the profile of the formation of immunoglobulin (Ig)G and IgM antibodies depending on the dose and duration of use is an urgent task of modern immunology. This is mainly due to the fact that the number of drug addicts continues to grow throughout the world, and the contingent of people using PSs such as antidepressants for non-medical purposes has also increased significantly. It should be taken into account the fact that new synthetic psychotropic drugs are developed annually, including and the so-called designer drugs, which at the initial stages of appearance on the market cannot always be identified by standard methods of chemistry due to changes in the structural formula.[1]

According to modern world standards, chemical and immunochemical methods for detecting the active substance of a psychotropic drug or its metabolites are used to

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determine drug intoxication.[2,3] In recent years, tests have been developed and introduced for the immunological determination of opiates, barbiturates, cannabinoids, and derivatives of ephedrine, which detect substances of PSs or their metabolites in a number of biological fluids. However, tests based on the direct detection of substances and their metabolites have a serious drawback, since the detectable concentration of a PS lasts for only few days, and the substance itself is rapidly metabolized and excreted.[4]

One of the available methods for detecting the use of PSs is the determination of specific antibodies to these substances. Although the overwhelming majority of PSs belong to the class of low molecular weight substances, nevertheless, when they enter the body, they partially bind to transport proteins of the blood, as well as receptors of some cells, thereby forming an immunogenic macrocomplex with the presentation of the hapten of a PS. Therefore, regular use of PS can lead to prolonged antigenic stimulation and the formation of specific Igs.

To date, there are few data in the literature on the effects of synthetic cannabinoids and antidepressants on the mammalian immune system. Immunological data from primary studies conducted in the 70s of the 20th century among people who abuse drugs and psychotropic drugs were very contradictory. Specific antibodies were detected only in a very limited number of drug addicts,[5-7] which is probably due, first of all, to the imperfection of detection methods. However, later studies have shown that, depending on a number of factors, the effectiveness of the detection of specific antibodies, such as opiates, can reach 70–80%.[8-10] The emergence of the possibility of detecting specific antibodies to PSs significantly expands the diagnostic significance of immunological methods for the detection of PSs, including synthetic cannabinoids and antidepressants. Moreover, modern studies show that the mechanism of the body's immunological responses to the administration of cannabinoids and antidepressants may vary. In this regard, when studying their immunogenicity, it is necessary to take into account the effectiveness of the formation of specific antibodies of the IgG and IgM class, depending on the duration and amount of PSs used.

The aim of this study was to study the formation of IgG and IgM class antibodies to synthetic cannabinoids and antidepressants to comparatively evaluate the role of the antibody class in detecting dependence on psychotropic drugs. For this purpose, conjugates for antidepressants were obtained: Faverin, carbamazepine, sertroline, fluoxetine, and for synthetic cannabinoids of the "spice" type – AB-FUBINACA and ADB-CHMINACA. The resulting conjugates were immunized mice according to the schemes for the induction of the formation of IgG and IgM antibodies.

Immunization efficiency was determined by comparing the increase in the level of optical density in enzyme-linked immunosorbent assay (ELISA) (at OD = 450 nm) in serum samples of immunized mice against the serum of control animals. In addition, the presence of a cross-reaction of the obtained antibodies with substances of the so-called endogenous neurotransmitters (adrenaline [epinephrine], norepinephrine [norepinephrine], and dopamine [dopamine]) was studied. A study of cross-heterologous immunological reactions between the studied PS compounds was also carried out, and the duration of circulation of specific antibodies after immunization was determined.

MATERIALS AND METHODS

Conjugates with four antidepressants were originally prepared: Faverin (E) -5-methoxy-1- [4- (trifluoromethyl) phenyl] pentan-1-one O-2-aminoethyl oxime, carbamazepine (5-carbamoyl-5H-dibenz- (6, f) azepine), sertroline ((1S, 4S) -4- (3,4-dichloro phenyl) -N-methyl-1,2,3,4-tetrahy dronaphthalen-1-amine), and fluoxetine ((RS) -N-methyl-3-phenyl-3- [4- (trifluoromethyl) phenoxy] propan-1-amine) and with two synthetic spice cannabinoids: AB-FUBINACA (N - [(1S) -1- (aminocarbonyl) -2-methylpropyl] -1 - (4-fluorophenyl) methyl] -1H-indazole-3-carboxamide) and ADB-CHMINACA (N - [(1S) -1- (Aminocarbonyl) -2-methylpropyl] -1- (cyclohexylmethyl) -1H-indazole-3-carboxamide) in conjunction with the carrier protein bovine serum albumin (BSA) (Sigma). The average hapten content in the conjugate was 14–16 mol.

Further, laboratory outbred white mice weighing 15–20 g were immunized with the resulting conjugates. One group of mice was immunized by a single intraperitoneal injection of the appropriate conjugate (five mice for each substance). When immunizing another group, the first injection was carried out intraperitoneally, followed by a double subcutaneous injection of a booster dose after 7 and 14 days (seven mice for each substance). In parallel, a control group of ten mice was formed, which was of the same age and weight as the experimental groups, but did not receive any drugs. At the end of immunization, decapitation of mice, collection of blood, and separation of serum in a standard manner were performed. Part of the serum was taken and frozen at –20°C. The remaining serum was used to enrich the antibody fraction by repeated ultrafiltration in ×1 PBS buffer with 150 FW ultrafilters (Amicon). In the obtained ultrafiltrates, the protein concentration was determined by biuret and the samples were aligned to a protein content of 2 mg/ml, and then 50 µl were aliquoted. Aliquots were stored at −20°C and used once as the "background" control used the serum of mice from the control group, not subjected to immunization. The control serum was obtained and prepared in the same way as the serum of immunized animals.

The prepared ultrafiltrates were tested for diagnostic efficiency in the recognition of the studied haptens of PSs

by ELISA with the corresponding haptens on the sorbing matrix. The amount of hapten applied for sorption was 300–500 ng per well, depending on the hapten. ELISA was performed on Linbro EIA Microplate polystyrene plates (ICN Biomed, USA). The analysis of ELISA results was carried out on a HUMAREADER-HS spectrophotometer (Human, Germany).

As the first antibodies for hybridization with haptens, the corresponding ultrafiltrates of the serum of immunized mice were used, as well as the ultrafiltrate of the control group in a 1:5000 dilution in the negative control well. As the second antibodies, the corresponding rabbit monoclonal antibodies to mouse IgG and IgM antibodies conjugated with peroxidase (Sigma) at a dilution of 1:10,000 were used according to the manufacturer's recommendation. The resulting immune complex was developed with a chromogenic substrate with tetramethylbenzidine (Sigma) in a buffer with hydrogen peroxide.[11] The spectrophotometer was used to determine the intensity of chromogenic staining at 450 nm with subtraction at 630 nm.

To identify cross-reactions among the obtained antibodies of mice immunized with various haptens, immunoblot hybridization was performed between a hapten of one of the substances adsorbed on a nylon membrane and various antibody samples of mice immunized with other substances of PS. For this purpose, serum samples of each group at a dilution of 1:1000 were used for immunoblotting against heterologous haptens sorbed on a nylon filter. A hypothetical immunocomplex was detected by a standard immunoblot procedure using rabbit anti-mouse polyclonal antibodies labeled with alkaline phosphatase (Santa Cruz Biotechnology, Inc.). The resulting immune complex was developed with a chromogenic BCIP/NBT substrate from the WesternBreeze Chromogenic Western Blot Immunodetection Kit (Invitrogen).

The determination of cross-reactions with neurotransmitters was determined by the hybridization of the obtained antibodies of mice with sorbed haptens of L-adrenaline, L-noradrenaline, and dopamine hydrochloride (Sigma).

To study the dynamics of circulation of specific antibodies, repeated immunization of new groups of mice (15 mice per group for each conjugate) was carried out according to the scheme described above with the introduction of booster doses, but five mice from the group were clogged 2, 4, and 8 weeks after the first immunization. Registration of the presence of antibodies was determined by ELISA under the above conditions.

RESULTS

The studies revealed the production of specific antibodies to IgG and IgM classes in mice immunized with four conjugates with antidepressants and two conjugates with synthetic cannabinoids. Most antibodies had low cross-reactivity with respect to natural neurotransmitters and heterologous haptens, with the exception of the studied "spices." It was shown that the circulation of specific antibodies is maintained at a detectable level for a sufficiently long time. Based on the results obtained, it can be concluded

Table 1: Data on the determination of antibodies to the studied synthetic cannabinoids and antidepressants by ELISA

Hapten	Class of detectable antibodies	The values of optical density in an enzyme immunoassay			
		With a single immunization*; av. OD =450	With repeated immunization**; av. OD = 450	Control group; av. OD = 450	Cut-off point
Faverin	IgM	0.530 (0.480–0.710)	0.741 (0.526–0.850)	0.150 (0.060–0.190)	0.280
	IgG	0.423 (0.400–0.538)	0.720 (0.560–0.820)	0.125 (0.065–0.140)	0.200
Carbamazepine	IgM	0.950 (0.820–1.115)	1.640 (1.130–1.700)	0.090 (0.020–0.100)	0.170
	IgG	0.785 (0.645–0.885)	1.307 (0.921–1.425)	0.110 (0.025–0.120)	0.205
Sertroline	IgM	0.710 (0.590–0.840)	0.930 (0.824–0.996)	0.065 (0.017–0.080)	0.130
	IgG	0.505 (0.440–0.695)	0.850 (0.733–0.890)	0.085 (0.021–0.110)	0.175
Fluoxetine	IgM	0.890 (0.760–0.940)	0.965 (0.830–1.102)	0.130 (0.040–0.160)	0.250
	IgG	0.470 (0.410–0.575)	0.773 (0.555–0.894)	0.080 (0.034–0.100)	0.150
AB-FUBINACA	IgM	0.812 (0.725–0.850)	1.010 (0.878–1.120)	0.142 (0.052–0.170)	0.260
	IgG	0.437 (0.405–0.563)	0.732 (0.516–0.841)	0.130 (0.048–0.152)	0.235
ADB-CHMINACA	IgM	0.951 (0.837–1.125)	1.185 (0.919–1.260)	0.135 (0.065–0.150)	0.220
	IgG	0.630 (0.531–0.835)	0.928 (0.817–0.990)	0.148 (0.095–0.180)	0.235

*Data on the hybridization of hapten and antibodies from a serum ultrafiltrate sample obtained from five mice immunized with the corresponding conjugate are presented. **Data are presented on the hybridization of hapten and antibodies from a sample of serum ultrafiltrate obtained from seven mice immunized with the corresponding conjugate. The ELISA was performed in six repetitions. The arithmetic mean values of optical density are given in six settings, and the extreme limits of variation in the values of optical density in the settings are given in parentheses. Av. OD 450 = average optical density at 450 nm. Calculation of cutoff = OPavK- + ΔK-. ELISA: Enzyme-linked immunosorbent assay. Ig: Immunoglobulin

Table 2: Immunoreactivity of mouse antibodies against heterologous haptens

Sorbed Hapten	The intensity of the chromogenic signal during the hybridization of heterologous haptens with the obtained antibodies to PS						The intensity of the chromogenic signal during hybridization of haptens with serum antibodies of mice from the control group (negative control)
	Antibodies to faverin	Antibodies to carbamazepine	Antibodies to sertroline	Antibodies to fluoxetine	Antibodies to AB-FUBINACA	Antibodies to ADB-CHMINACA	
Faverin	x	45	60	53	130	135	143
Carbamazepine	80	x	113	60	142	51	211
Sertroline	112	116	x	520	125	86	200
Fluoxetine	106	121	645	x	83	115	132
AB-FUBINACA	92	104	82	58	x	9 115	127
ADB-CHMINACA	78	98	110	130	7 205	x	161

x is a combination of a homologous pair of hapten antibodies

that the detection of IgM antibodies is the most prognostically significant for the diagnosis of PSs, and further study of the selection of conditions for the detection of specific antibodies and the study of the dependence of PS immunogenicity on the structure will significantly enhance the immunological detection of synthetic cannabinoids and antidepressants.

DISCUSSION

In our work, to increase knowledge on the formation of antibodies to synthetic cannabinoids and antidepressants, mice were immunized to stimulate the synthesis of antibodies of two classes: IgM and IgG. In this regard, immunization was carried out according to two schemes: By a single intraperitoneal administration and by repeated administration of a booster dose to stimulate IgG antibodies. In immunization, conjugates of PV haptens with BSA as a carrier protein were used. Mice were immunized with the obtained conjugate, from the total serum of which the protein fraction enriched in antibodies was subsequently isolated by ultrafiltration. The working titer of antibodies was determined by dot blot directly on the nylon membrane. For most substances, it was 1:5000 and was used in further ELISA studies in this dilution. Further, by ELISA, the production of IgM and IgG antibodies to PS was detected in mouse samples. As a result of ELISA, the following indicators were obtained [Table 1].

From the above values, it can be concluded that the presence of specific antibodies to the test substances was established in the blood serum of immunized mice. However, the severity of antibody formation or antibody immunoreactivity probably varies depending on the class of Igs and the nature of the hapten in the conjugates used in immunizing mice. It is important to note that the same BSA was used as the carrier protein for the conjugate. In this regard, we can say that the immunogenicity of the conjugate, ceteris paribus, is due precisely to the nature of the PS hapten itself.

Hence, in relation to antidepressants, the most pronounced formation of antibodies of both classes is observed during immunization with carbamazepine and fluoxetine. It is important to note that in the general case, the immunoreactivity of IgM antibodies is higher compared to the immunoreactivity of IgG antibodies. However, IgM antibodies are decavalent (it has five binding rays), in contrast to divalent IgG antibodies, which possibly leads to a wider spectrum of recognition of haptens by IgM antibodies, the efficiency, and stability of the formation of the immune complex *in vitro*, therefore, it is correct to designate exactly manifestation of increased immunoreactivity of IgM antibodies in relation to the studied haptens of PS. At the same time, it should be noted that the immunoreactivity of antibodies to the ADB-CHMINACA spice is higher than in the case of the AB-FUBINACA related spice.

The cross-reactions between the obtained antibodies of mice and the studied haptens were also determined in the work.

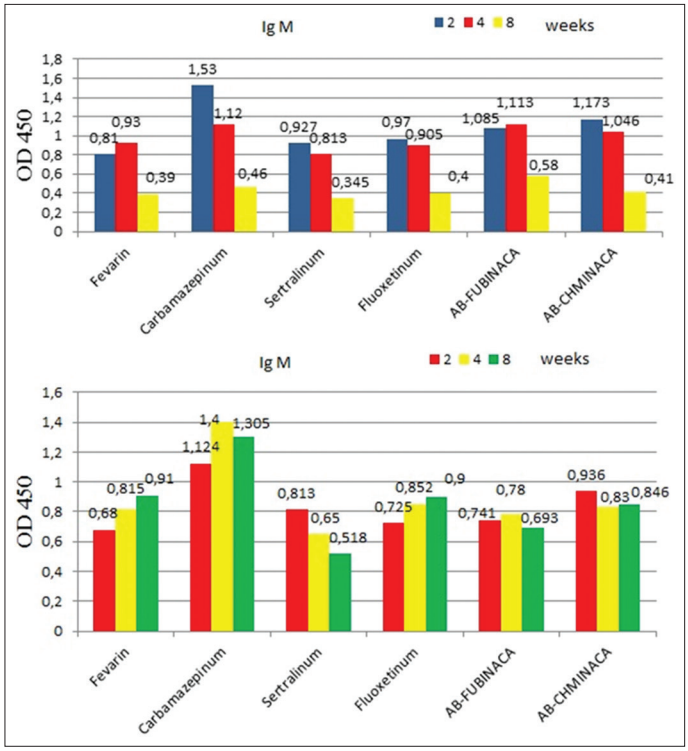

Figure 1: Schedule changes in the registration of antibodies of immunoglobulin (Ig)M-class (I) and IgG-class (II) depending on the time elapsed after immunization

Reactivity was determined by the presence or absence of chromogenic bands on the immunoblot membrane. The intensity of the chromogenic replica was evaluated by spot densitometry [Table 2]. As a result of studying the cross-reactivity of total antibodies from the blood serum of immunized mice, it was found that moderate cross-reactivity is recorded between heterologous haptens such as fluoxetine and sertroline, as well as pronounced cross-reactivity in the case of both spices. The effect of cross-reactivity, the so-called natural neurotransmitters, and antibodies specific for PS, was studied separately. In this case, pronounced cross-reactivity has not been established. The circulation dynamics of specific antibodies on repeated immunization of new groups of mice showed that the mice retained the production of specific IgM-class antibodies for at least 2–4 weeks and IgG-class antibodies for at least 4–8 weeks from the time of drug administration [Figure 1].

CONCLUSION

In this research, antibodies to low-molecular substances, in particular to synthetic cannabinoids "spice" and antidepressants were obtained, it was shown that during circulation, specific antibodies at the detectable level remain for a long time. It was found that the most prognostically significant for the diagnosis of PS use is the detection of IgM class antibodies.

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