The Spectrophotometric Characteristic of Immunoglobulin Conjugates for Diagnostics of Causative Agents of Especially Dangerous Infections

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Abstract—The possibility of characterizing fluorescent immunoglobulins using spectrophotometric analysis as a testing method is considered. The comparative analysis of optical properties of fluorescent immunoglobulin preparations and their components—immunoglobulins and fluorochrome—is carried out. The obtained results testify that the proposed methodological approach of optical detection of labeled immunoglobulin molecules can be promising for tests on obtaining conjugates used in immunological tests on revealing specific antigens of causative agents of especially dangerous infections.

Keywords: spectrophotometry, immunoglobulins, conjugates, fluorescein isothiocyanate

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INTRODUCTION

The possibility of adding fluorescent molecules to antibodies was proposed for the first time by Albert Coons in 1942 [1]. This researcher applied a method that he developed to detection of the pneumococcal antigen in tissues. The method, called the “fluorescent antibody method” (FAM) or “immunofluorescence,” is based on using the specificity of the immunological response and sensitivity of luminescent (fluorescent) microscopy [2]. At present, the FAM is widespread as an important component in biological and medical investigations, in particular, for the specific indication of causative agents of especially dangerous infections.

A distinction is made between the direct FAM (dFAM) developed by Coons and Melvin Kaplan [3] and indirect FAM (iFAM) proposed by Thomas Weller and Coons [4]. In the direct method, a known serum with fluorescent antibodies supposedly corresponding to the sample under study—a specimen with the antigen—is deposited on the sample. In the case of the formation of the antigen—antibody complex, the attached antibodies are detected by luminescent microscopy in the form of a fluorescent signal with different degree of intensity determined visually by the researcher and depending on the number of fluorochrome-labeled antibodies attached to the cell. When performing indirect analysis, an immune serum non-labeled to the sought causative agent is deposited on the antigen. In the case in which the serum antibodies correspond to the antigen, an antigen—antibody immune complex is formed. Then, an antispecies serum with fluorochrome is deposited on the preparation. Thus, antibodies of the first serum serve as an antigen for labeled antibodies of the antispecies serum. The forming double complex is determined using a luminescent microscope. The indirect method is more universal because using a single fluorescent serum allows one to reveal different species of microorganisms.

Antibodies labeled by a fluorescent dye preserve the ability to enter into a specific reaction with a homologous antigen the position of which is determined using luminescence microscopy from the characteristic luminescence appearing after the excitation of the fluorescent agent by ultraviolet radiation. The main requirements to fluorochromes used for labeling of specific proteins are the distinguishability of their fluorescence color from the autofluorescence of the object under study, contrasting with the background, high fluorescence intensity after conjugation with the protein, and preservation of main physicochemical and serological properties of antibodies. For the fluorochrome, fluorescein isothiocyanate (FITC) [5, 6], rhodamine sulfonyl chloride (RSC) [7, 8], rhodamine sulfonyl fluoride (RSF) [9, 10], tetramethyl-
rhodamine isothiocyanate (TRITC) [11, 12], dichlorotriazinylaminofluorescein (DCTAF) [13, 14], etc., are used. Such organic compounds emit yellow-green, yellow, and red fluorescence. The dye that is most often used as a label of antibodies is FITC. This dye, the molecular formula of which is C$_{21}$H$_{11}$NO$_5$S, causes a green fluorescence; it bonds covalently to biomolecules via the NCS functional group and has a high quantum yield [15, 16]. The maximums of the absorption and fluorescence wavelengths are 495 and 520 nm, respectively [17]; owing to this, the background fluorescence of biological specimens is reduced to the minimum.

Traditionally, preparation of conjugated antibodies is controlled using the fluorescent antibody method. It is also recommended to determine the dye/protein molar ratio for each conjugate by optical methods. In the absorption spectrum of a labeled antibody, the dye peak and the absorption peak of the antibody are present. To calculate the degree of labeling (the number of fluorochrome molecules introduced per one molecule of the antibody), it is necessary to measure the optical density of the solution at the absorption maximum of the antibodies (A$_{AB}$) and wavelength of the absorption maximum of the dye (A$_{Dye}$). The number of fluorochrome molecules per one molecule of the antibody is calculated [18] by the formula

\[
\frac{D_{Dye} \varepsilon_{AB}}{AB} = \frac{A_{Dye} \varepsilon_{AB}}{(A_{AB} - A_{Dye} \cdot CF) \varepsilon_{Dye}}, \tag{1}
\]

where \(D_{Dye} \varepsilon_{AB}/AB\) is the sought number of dye molecules per an antibody molecule, \(A_{Dye}\) is the optical density of the sample at the wavelength of the absorption maximum of the dye, \(A_{AB}\) is the optical density of the sample at the wavelength of the absorption maximum of the proteins (280 nm), \(\varepsilon_{AB}\) is the molar extinction coefficient of the antibody at the wavelength of 280 nm (for IgG, 210000), \(\varepsilon_{Dye}\) is the molar extinction coefficient of the dye at the wavelength of the absorption maximum (for FITC, 72000 M$^{-1}$ cm$^{-1}$), and CF$_{280}$ is the correction factor for the dye at the wavelength of 280 nm.

Protein concentration \(C\) and the dye–protein molar ratio \((F/P)\) of conjugates is determined approximately by measuring the optical density \((A)\) at different wavelengths [2] according to the formulas

\[
C \text{ (mg/mL)} = \frac{A_{280} - (0.35A_{495})}{1.4}, \tag{2}
\]

\[
\frac{F}{P} = \frac{2.87A_{495}}{A_{280} - (0.35A_{495})}. \tag{3}
\]

At the optimum relation \(F/P\),

\[
0.5 < \frac{A_{495}}{A_{280}} < 1.5.
\]

The optimum degree of labeling to obtain a good fluorescent signal is in most cases two or three dye molecules per antibody molecule. The further increase in the labeling degree does not lead to a significant enhancement of the fluorescent signal because the concentration quenching of fluorescence is observed. If the labeling degree is insufficient, it is necessary to decrease the quantity of the antibody introduced into the reaction.

**OBJECT OF THE WORK**

The objects of the work are as follows:

— to estimate the possibility of using spectrophotometric analysis for control of obtaining fluorescent immunoglobulins; and

— to determine criteria of fluorescent dye binding to antibodies by the example of FITC.

**MATERIALS AND METHODS**

The optical scheme for carrying out the spectrophotometric analysis includes

—an AvaLight-HAL compact light source (Avantes, Netherland) for measurements in a spectral wavelength range from 360 to 1700 nm;

—an HR4000 high resolution spectrometer (Ocean Optics, United States) for recording spectral characteristics of samples under study in a wavelength range from 200 to 1100 nm;

—fiber-optic cables (Ocean Optics, United States); and

—a cuvette holder (Avantes, Netherland); and

—UVette plastic spectrophotometric cuvettes (Eppendorf, Germany) for measurements in a wavelength range from 220 to 1600 nm.

In the work, we used

— equine diagnostic adsorbed fluorescent plague immunoglobulins and lyophilizate for diagnostic purposes (Mikrob Russian Antiplague Research Institute, registration certificate no. FSR 2007/0881-240409) in the form of the fluorescein-5-isothiocyanate (FITC) labeled immunoglobulin fraction of the plague agglutinating serum;

— dry diagnostic fluorescent tularemia immunoglobulins (Gamaleya Scientific Research Institute of Epidemiology and Microbiology, registration certificate no. FSR 2010/08923) in the form of the fluorescein-5-isothiocyanate (FITC) labeled globulin fraction obtained from the equine hyperimmune tularemia serum;

— equine diagnostic adsorbed fluorescent plague immunoglobulins for the agglutination reaction on glass and lyophilize for diagnostic purposes (Mikrob Russian Antiplague Research Institute, registration certificate no. FSR 2008/02592-270409);
—dry diagnostic tularemia serum for the agglutina-
tion reaction (Irkutsk Antiplague Research Institute,
registration certificate no. FSR 2011/10029); and
—fluorescein-5-isothiocyanate (FITC) (Sigma-
Aldrich F7250-5G, United States).

CONJUGATION
OF IMMUNOGLOBULINS WITH FITC

The immunoglobulin concentration was brought up
to 2% in protein, and FITC was added in an
amount of 2–3 mg per 100 mg of protein. The conjuga-
tion process occurred in a refrigerator for 18 h under
continuous mixing on a magnetic shaker.

FITC not bound to protein was removed
chromatographically using a G-25 Sephadex column.

To remove fluorochrome that is chemically not
bound to protein, the obtained conjugate was depos-
ted on a prepared chromatographic column prelimi-
narily equilibrated by 0.15 M sodium chloride solution
containing 5% of carbonate-bicarbonate buffer
(CBB). The same buffer is used for eluting the labeled
protein from the column.

The activity of the prepared conjugate was tested by
luminescence microscopy of bacterial cells of corre-
sponding infections. The specificity was revealed by
microscopy of cells of other bacterial species.

EXPERIMENTAL MEASUREMENTS

Spectral characteristics of fluorescent plague and
tularemia immunoglobulins labeled with FITC, non-
labeled immunoglobulins and FITC, and a mixture of
FITC with nonlabeled immunoglobulins were spec-
trophotometrically analyzed and recorded using an
HR4000 spectrometer according to the instructions
for use [19].

Before starting the measurements, the reference
and dark spectra were preserved in the control soft-
ware of the Spectra Suite spectrometer (Ocean Optics,
United States) to exclude the spectrum of the solvent
(physiological salt solution) and external light radia-
tions. For this purpose, 100 μL of physiological salt
solution were poured into a clean cuvette, the cuvette
was placed in a cuvette holder, and the reference and
dark spectra were recorded when the light source was
powered on and off, respectively. Then, the spectrum
of light passing through the sample was recorded in ten
repetitions according to the user manual supplied with
the device. The used software allows one to take into
account the possibility of reabsorption and the prefil-
ter effect; the maximum value of the optical density
measured by the device is 3.8. The optical density
(light absorption) of the samples under study at each
wavelength is calculated in the Spectra Suite software
by the following formula and represented as a spec-
trum plot:

\[ A_\lambda = -\log_{10} \left( \frac{S_\lambda - D_\lambda}{R_\lambda - D_\lambda} \right), \]  \hspace{1cm} (4)

where \( S_\lambda \) is the analytical signal at wavelength \( \lambda \), \( D_\lambda \) is
the dark signal at wavelength \( \lambda \), and \( R_\lambda \) is the reference
signal at wavelength \( \lambda \).

Spectra of each sample were exported as tables to
the Origin™ software (OriginLab Corp., United
States) for further analysis.

RESULTS AND DISCUSSION

In the course of the experimental work, absorption
spectra of plague and tularemia immunoglobulins and
labeled FITC, as well as absorption spectra of FITC
and nonlabeled immunoglobulins in isolation from
one another, in addition to the absorption spectrum of
a mixture of nonlabeled immunoglobulins and FITC,
were obtained.

It has been found that the FITC solution in alcohol
had an absorption maximum at a wavelength of
495 nm, which agrees with the literature data [20, 21],
and an absence of absorption at wavelengths above
560 nm (Figs. 1a, 2a). The absorption spectra of non-
labeled plague and tularemia immunoglobulins had
peak values in the region of 380–450 nm with a grad-
ual falloff in the longwave region due to light scattering
of protein solutions (Figs. 1a, 2a). The light scattering
curve of protein solutions on the wavelength interval
of 500–850 nm was described with a high degree of
approximation by the equation proposed by W. Heller
et al. [22] for describing the light scattering of spheri-
cal particles (Fig. 3):

\[ A_{sc} = \beta \lambda^{-n}, \]  \hspace{1cm} (5)

where \( A_{sc} \) is the optical density caused by the light scat-
tering, AU; \( \lambda \) is the wavelength, nm; and \( \beta \) and \( n \) are
coefficients.

Mixing the FITC solution with nonlabeled immu-
oglobulins, we obtained the resulting absorption
spectrum (Figs. 1b, 2b) equal to the mathematical sum
of the absorption spectra of FITC and nonlabeled
immunoglobulins. This indicates the absence of the
interaction between the components constituting the
mixture at the instant of mixing and the absence of
other absorbing components in the mixture.

The conjugation of protein with fluorochrome is a
chemical reaction resulting in the formation of a new
compound in which the dye is bound to protein by a
covalent bond; as a consequence, the optical (spectral)
characteristic of the obtained conjugate changes
(Figs. 1b, 2b).

The spectral curves of fluorescent immunoglobu-
lin after conjugation with FITC followed by purifica-
tion had two clearly pronounced maximums of the
One of the maximums corresponded to absorption of antibodies in the range of 380–450 nm, but the absorption was shifted to the shortwave region by 50 and 20 nm and reduced by 60‒70% in the amplitude. The second maximum corresponded to absorption of the fluorescent label at the wavelength of 495 nm (Figs. 1b, 2b).

CONCLUSIONS

The performed investigations have resulted in obtaining the spectral characteristics of plague and tularemia immunoglobulins labeled with FITC. Studying the absorption spectra of fluorescent immunoglobulins demonstrates the presence of two absorption maximums: at a wavelength of 495 nm corresponding to the fluorescent label and at a wavelength of 380 nm characteristic for nonlabeled immunoglobulins. In contrast to the absorption spectrum of serums absorption spectrum.
without fluorescent labels, the characteristic absorption peak of antibodies was positioned closer to the shortwave band by 50 and 20 nm; in addition, a decrease in the absorption intensity by 60–70% on average was observed in this spectral range. The presence of two absorption peaks in fluorescent immunoglobulins is a criterion of the difference between prepared conjugates and a mixture of nonlabeled immunoglobulins and FITC and can be used for preliminary control of the conjugate synthesis at the stage of production of fluorescent immunoglobulins.

Thus, based on the obtained experimental data, one can conclude that absorption spectrophotometry makes it possible to distinguish fluorescent immunoglobulins obtained as a result of chemical conjugation of immunoglobulins with the dye from antibodies not bound to fluorochrome by the absorption spectrum. It can be used for the control for the synthesis of fluorescent conjugates in addition to luminescent microscopy.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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