Original article

Do Water Molecules Displaced by Hydrophobic Interactions Stabilize Antigen-Antibody Binding? Physico-chemical background of antigen-antibody reactions analyzed by fluorescent and Fourier-transform infrared spectroscopy on FITC – anti-FITC (IgG1) model

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Abstract

Background: Antigen-antibody reactions are a special field of molecular interactions. The physico-chemical nature of antigen-antibody binding and ligand-induced changes in the fine molecular structures of antigens during immunocomplex formation are less studied. However, these changes in the molecular appearance are extremely important for further molecular recognition. The major aim of this study is to clarify the physico-chemical modification of the antigen/hapten during immunobinding using model experiments.

Methods: An appropriate model system was designed for our investigations: fluorescein-isothiocyanate (FITC, isomer I) was used as the antigen (hapten), and its interactions with a specific antibody (monoclonal anti-FITC IgG1) were analyzed using spectrophotometry, different spectrofluorimetric methods and fluorescence polarization, and Fourier-transform infrared spectroscopic methods.

Results: Fluorescent polarization and infrared spectroscopic measurements detected a local decrease in the hydration degree in the submolecular area of the specific ligand between the small antigen (hapten) molecule and the hypervariable region of the specific IgG1, causing "rigidization" of molecular movements. Changes in hydration modified the molecular microenvironment, allowing them to influence further functions of both immunoglobulins and the antigen.

Conclusion: Hydrophobic interactions with exclusion of water molecules around the binding sites seem to be thermodynamically strong enough for stable molecular binding without a covalent chemical interaction between the antigen and the antibody. The results of this study, together with data obtained in previous research, help understand the molecular dynamics of the antigen-antibody reaction better.

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Introduction

Antigen-antibody interactions are special forms of molecular associations in vivo and in vitro. The influence of antigen molecules on specific immunoglobulins during the formation of immune complexes has been studied: modification of the physico-chemical character of immunoglobulins after the binding of an antigen can influence the biological functions of an antibody (e.g. complement fixation, Fc receptor functions, etc.) (1, 2, 3, 4). However, our knowledge about diverse modifications of the fine molecular structure of antigens, especially changes in the structure of low molecular weight antigens (or so-called haptens) by specific immunoglobulins, is limited (5, 6, 7). However, these physico-chemical changes in the fine structure of antigens cross-linked by immunoglobulins seem to be important in the further regulation of several other molecular interactions. Activation or inhibition of different receptors, enzymes, and transporter molecules interchange during immunocomplex formation. Both new physiological and pathological immune reactions occur by the modification of the primary physico-chemical appearance of the antigenic structure after binding with an immunoglobulin (8). The submolecular mechanism of immunobinding and consequential modifications of fine molecular structures are poorly understood (9, 10, 11). Simple electrostatic coherence between the antigen and immunoglobulins is not thermodynamically strong enough to form a stable molecular complex and cause remarkable modifications of physico-chemical appearances of participating molecules (12, 13).

An analysis of hapten-immunoglobulin interactions is more favourable for studying the physico-chemical nature of structural modifications of the antigen caused by immunobinding. We have developed an experimental model system that allows us to use spectrophotometry, spectrofluorimetry, different fluorescence polarization techniques, and Fourier-transform infrared spectroscopy (FT-IR) as delicate methods for a complex analysis of the physico-chemical nature of hapten-immunoglobulin interactions. A conventional fluorescent dye (FITC) was used as the antigen and a specific monoclonal antibody (anti-FITC IgG1) against this hapten molecule had been developed at our department earlier (14). Immunological recognition and the consequential physico-chemical changes in the antigen/hapten structure were studied by carrying out a comparative analysis of native and chemically cross-linked FITC molecules. The further aim of this study was to find theoretical explanations for the feature of strong molecular interactions between the antigen and antibody, which are thermodynamically equivalent to a covalent chemical bond, without a real chemical ligand formation (15).

Materials and Methods

Antigen:

Fluorescein-iso-thiocyanate (FITC, isomer I) was used as a hapten (Mw: 389.4, product of the Sigma Chemical Company, USA), and its interactions with a specific antibody were studied using spectrophotometry, different spectrofluorimetric methods, fluorescence polarization, and Fourier-transform infrared spectroscopic methods.

For the preparation of the standard antigen stock solution, 1 mg FITC was dissolved in 100 µl dimethyl-sulfoxide (DMSO, Sigma Chemical Company, USA), then diluted to 1 mg/ml concentration with PBS 0.15 M, pH 7.2 (phosphate buffered saline containing 5.4 mM Na2HPO4, 1.5 mM KH2PO4, 140 mM NaCl and 2.7 mM KCl). This stock solution was kept at 4 °C.

For the comparative analysis of free (native) FITC and the carrier cross-linked form, different protein molecules were labelled with FITC as bovine serum albumin (BSA, Sigma Chemical Company, USA), polyclonal anti-rat-IgG antibody (produced in sheep by our laboratory), and monoclonal mouse antibodies with IgG1 isotype (anti-FITC, anti-β hCG, anti-insulin produced by our department earlier). The FITC-labelling of proteins was carried out by following the method described (17). After protein labelling, the free, non-conjugated FITC

was removed from the reaction mixture by gel-filtration on a Sephadex G 25 FPLC column (Pharmacia, Sweden) equilibrated with PBS. Afterwards, the so-called conjugation rate – that is, the number of FITC molecules coupled onto one protein molecule – was determined semi-quantitatively by measuring the optical density of solutions of labelled proteins at 495 and 280 nm and by applying an empirical formula for the calculation:

$$X = \frac{2.87 * A_{495}}{A_{280} - 0.35 * A_{495}}$$

where x is the conjugation rate and A280 and A495 are the optical density of the solution measured at 280 and 495 nm, respectively (18).

The conjugation rate was 10.7 for FITC-BSA, 8.6 for FITC-anti-rat-(sheep) IgG, 12.4 for anti-FITC IgG1, 10.3 anti-βhCG, and 11.3 for anti-insulin IgG1 antibodies in our experiments.

Antibody:

The monoclonal anti-FITC antibody (mouse, IgG1) was developed at our department earlier (14). The mass of the antibody was produced by hybridoma fermentation using Harvestmouse (Serotec, UK) hollow-fibre fermenter and affinity-purified on a FITC-BSA-coupled Sepharose 4B column. The column was prepared as usual (16). In brief, 1.8 g of CNBr-activated Sepharose 4B (Pharmacia, Sweden) gel was suspended, swollen and washed five consecutive times in 50 ml 1 mM HCl. 100 mg BSA (Sigma Chemical Company, USA) was dissolved in 10 ml coupling buffer, that is 0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl, and mixed with the gel. The mixture was being rotated end-over-end for two hours at room temperature. Following a thorough wash in the coupling buffer, the gel was left to react with 300 µg/ml FITC, generally following the FITC-labelling method described above (17). Afterwards, the reaction was stopped and all remaining active groups were blocked by washing in 50 ml 0.1 M pH 8 Tris-HCl buffer for two hours at room temperature. The gel was then washed in five cycles of alternating pH. Each cycle consisted of a wash with 0.1 M pH 8 acetate buffer containing 0.5 M NaCl. Following this washing procedure, the gel – adding up to 7 ml – was poured into a column and equilibrated with PBS containing 0.05% NaN_3.

The anti-FITC antibody was purified on the aforementioned column, thus ensuring that all antibody molecules would recognize the hapten selectively. 800 ml hybridoma supernatant was flown slowly through the column for 24 hours at 4 ˚C. The antibody was then eluted from the column with 0.1 M pH 2.5 glycine buffer. 1-ml fractions were collected and 100 µl 1 M pH 9 Tris-HCl was added instantly to each of them. Afterwards, the concentration was determined by measuring the optical density at 280 nm.

The purified monoclonal anti-FITC antibody was fragmented to Fab using papain as usual (19). The purified anti-FITC antibody was digested with papain in the presence of a reducing agent, cysteine. To determine optimal conditions, pilot fragmentation was carried out, and both the concentration of papain and the time of digestion were varied. After the pilot fragmentation, the fragments were dialyzed and analyzed by PhastSystem SDS-PAGE fast gel electrophoresis (Pharmacia-LKB, Sweden). Optimal conditions were determined according to the gel results. These conditions were used in the large-scale fragmentation of anti-FITC to anti-FITC Fab. 5 ml anti-FITC IgG1 monoclonal antibody (with a concentration of 2 mg/ml) was added to a freshly-mixed digestion buffer (PBS 0.15 M, pH 7.2, 0.02 M EDTA, 0.02 M cysteine), containing 0.1 mg/ml papain. The enzyme-antibody ratio was 1:20. The reaction mixture was well-mixed and incubated in a water bath at 37 ˚C. After six hours, the mixture was removed from the water bath, and 1 ml of 0.3 M iodoacetamide in PBS was added to stop the reaction. The reaction mixture was dialyzed against 2 litres PBS, pH 8.0, for 24 hours at 4 ˚C. The products were analyzed with Pharmacia's PhastSystem SDS-PAGE fast electrophoresis.

A 10 x 200 mm protein A-Sepharose CL-4B (Pharmacia, Sweden) column was made, and the

dialyzed reaction mixture was loaded onto it. Unbound fractions containing the Fab fragment and enzyme were collected and the column was washed with PBS to remove the Fab fragments completely. The mixture was concentrated to 5 ml. A 26 x 900 mm Sephacryl S-200 Superfine column (Pharmacia, Sweden) was made and the concentrated reaction mixture was loaded onto it. Fractions of 50 kD were collected and the purity of the final product was checked using 10% nonreducing SDS-polyacrylamide gel (Pharmacia's PhastSystem). The concentration of the Fab fragments was assessed at A280 and stored in borate buffer (0.015 M sodium borate, 0.15 M NaCl, pH 8.5) at 4 ˚C.

We used monoclonal anti-βhCG antibody (mouse, IgG1) as an isotype (negative) control, which had also been developed at our department earlier. The production, purification, labelling, and preparation of the Fab fragments were carried out as described in case of the anti-FITC IgG1 monoclonal antibody.

Spectrophoto- and fluorimetry, FT-IR spectroscopy:

In order to avoid the so-called "inner filter effect" (this phenomenon can be seen when a solution of a given compound is concentrated enough to absorb an already significant ratio of the exciting light beam, thus reducing light intensity at the centre of the cuvette where the emitted light is detected, consequently interfering with the results), prior to fluorimetric measurements, all samples were diluted in PBS 0.15 M pH 7.2 to a concentration at which the optical density of the solution measured at its absorption and emission peaks was below 0.05. This concentration was 50 ng/ml for free FITC, 5 µg/ml for FITC-BSA, 10 µg/ml for FITC-labelled anti-rat-IgG, and 10 µg/ml for monoclonal antibodies with IgG1 isotype.

Non-diluted protein concentrations were determined by measuring the optical density at 280 nm for all immunoglobulins and by using the dye-binding assay for BSA as described (20, 21).

Spectrophotometric measurements were performed on a UV/VIS photometer (DU-68, Beckman Instruments Inc., USA) using 2 x 10 mm quartz cuvettes, all at room temperature.

Some fluorescence-quenching measurements were done on a steady-state fluorimeter (Locarte, England), in cylindrical cuvettes of 3 mm in diameter, at room temperature. A 330 W Zn arc lamp was used as the excitation light source. 490 nm excitation wavelength was selected using the LF3 monochromatic filter. Emission was monitored through LF4 and LF7 cutoff filters and the 510-570 nm range was therefore selected.

The majority of steady-state and all spectrofluorimetric measurements were performed on a Hitachi-Perkin Elmer (MPF 4) spectrofluorimeter equipped with polarization filters and a thermostated cell holder in 10 x 10 mm quartz cuvettes operated in ratio mode, all at room temperature. The light source was a 300 W Xe arc lamp. Both excitation and emission wavelengths were set by monochromators and the precise values were dependent on the actual fluorescent solution. Slits were 4 and 8 nm, respectively.

Fluorescence polarization measurements were achieved on the Hitachi-Perkin Elmer (MPF 4) spectrofluorimeter. With each sample, four measurements of fluorescence intensity were taken using one pair of polarization filters. The first sample was taken using vertically polarized exciting light and detecting vertically polarized emitted light (Ivv), the second included vertical excitation and horizontal emission (Ivh), the third involved horizontal excitation and vertical emission (Ihv), and the fourth sample included horizontal excitation and horizontal emission (Ihh). Following these measurements, the fluorescence anisotropy of FITC or FITC-labelled proteins could be calculated using an appropriate equation (see below).

Fluorescence life span measurements were taken on an ISS multifrequency phase fluorimeter (ISS Fluorescence Instrumentation, Champaign, Illinois, USA) using the frequency cross-correlation method. A 300 W Xe arc lamp was used as the excitation light source. Excitation light intensity was modulated using a

double-crystal Pockel cell and a two-way polariser. A cross-correlation frequency of 80 Hz was used. The excitation wavelength was set to 493 nm and the emission was also monitored through a monochromator set to 512 or 522 nm. A freshly prepared glycogen (Sigma Chemical Co., USA) solution was used as a reference to correct the instrumental phase delay of the detection equipment. Ten sets of phase and modulation data were collected for each sample. Phase and modulation data were analyzed using the ISS 187 Decay Analysis Software.

Fluorescence anisotropy was calculated using the following equation:

$$r = \frac{(vv - vv_S) - G(vh - vh_S)}{(vv - vv_S) + 2G(vh - vh_S)}$$

where r is fluorescence anisotropy; vv and vh are the fluorescence intensity measured with polarization filters set vertically in the excitation and vertically or horizontally in the emission light beam, respectively; vvs and vhs are the fluorescence intensity of control (FITC) samples measured with polarization filters set vertically in the excitation and vertically or horizontally in the emission light beam, respectively; and G is a correction factor, which is calculated using the following equation:

$$G = \frac{hv - hv_S}{hh - hh_S}$$

where hh and hv are the fluorescence intensity measured with polarization filters set horizontally in the excitation and horizontally or vertically in the emission light beam, respectively; hhs and hvs are the fluorescence intensity of control (FITC) samples measured with polarization filters set horizontally in the excitation and horizontally or vertically in the emission light beam, respectively. (22, 23)

The Perrin equation was applied to analyze fluorescence anisotropy of a given fluorophore depending on its molecular microenvironment:

$$\frac{1}{r} = \frac{1}{r_0}\left(1 + \frac{\tau_F}{\tau_D}\right)$$

where r and r0 are anisotropy and so-called limit anisotropy – the anisotropy extrapolated to 0 K temperature; tF and tD are fluorescence lifetime and rotation correlation time (22, 23).

Fourier-transform infrared spectroscopy (FT-IR) measurements were taken on a Spectra 400 FT-IR spectrometer (Nicolet, USA). 1-mg samples were lyophilized for 24 hours in a Savant Speed Vac lyophilizer (Speed Vac Plus SC210A, Savant, USA). The lyophilized samples were desiccated further for 68 hours in a vacuum desiccator filled with silica crystals. 0.1-0.5 mg of the dehydrated samples were pastillized in KBr. The Fourier-transform infrared transmittance spectrum was measured at a range of 400-4000 cm-1 and studied with a 4 cm-1 resolution. During the investigations of samples, the IR spectra of water vapor, carbon dioxide, and water of potassium bromide were subtracted from the total numeric data before the assignation. The IR bands were identified according to data in the relevant literature (24, 25, 26, 27).

For the analysis of solvent polarity, acetone was used in different dilutions as a non-polar solvent. FITC was dissolved in DMSO, as usual, but afterwards it was diluted in mixtures of acetone and water in various ratios. These samples were measured in cuvettes covered with a lid to prevent the acetone from evaporating. The fluorescence intensity and light absorbency was detected in a thermostatic fluorimeter at 20 °C.

Florescent spectroscopic measurements were repeated from two up to ten times independently and the scattering between the results was under 3% in all cases.

Results

The basic finding was that FITC fluorescence intensity definitely suffers from a fluorescence-quenching effect following pre-incubation with our anti-FITC IgG1 monoclonal antibody (12). Similar observations were made in case of using polyclonal and monoclonal (high affinity IgM) antibodies against FITC (9, 28, 29). Our model permits kinetic studies with different fluorescent

and infrared spectroscopic measurements by the advantageous structure of the mouse IgG1 molecule and its Fab fragment. During the optimization of fluorescence quenching measurements, FITC with anti-FITC antibody were incubated together for 1 - 10 minutes at room temperature in order to let the equilibrium be established, and then the fluorescence intensity of the reaction mixture was quantified. A trial to measure the time needed for anti-FITC to fully achieve its fluorescence-quenching effect was performed. We added a suitable amount of anti-FITC to a cuvette with the FITC solution already inside the fluorimeter and tried to monitor the decline of fluorescence intensity – with the results verifying our assumptions, since there was an instant quenching of fluorescence. According to our pilot study, the optimum incubation time for maximum fluorescence quenching caused by the anti-FITC monoclonal antibody was 10 minutes. No similar fluorescence-quenching effects were found when FITC was preincubated by non-specific monoclonal antibodies with the same isotypes.

We found total (100%) inhibition of FITC fluorescence intensity induced by both anti-FITC monoclonal antibody and its Fab fragment (Figure 1).

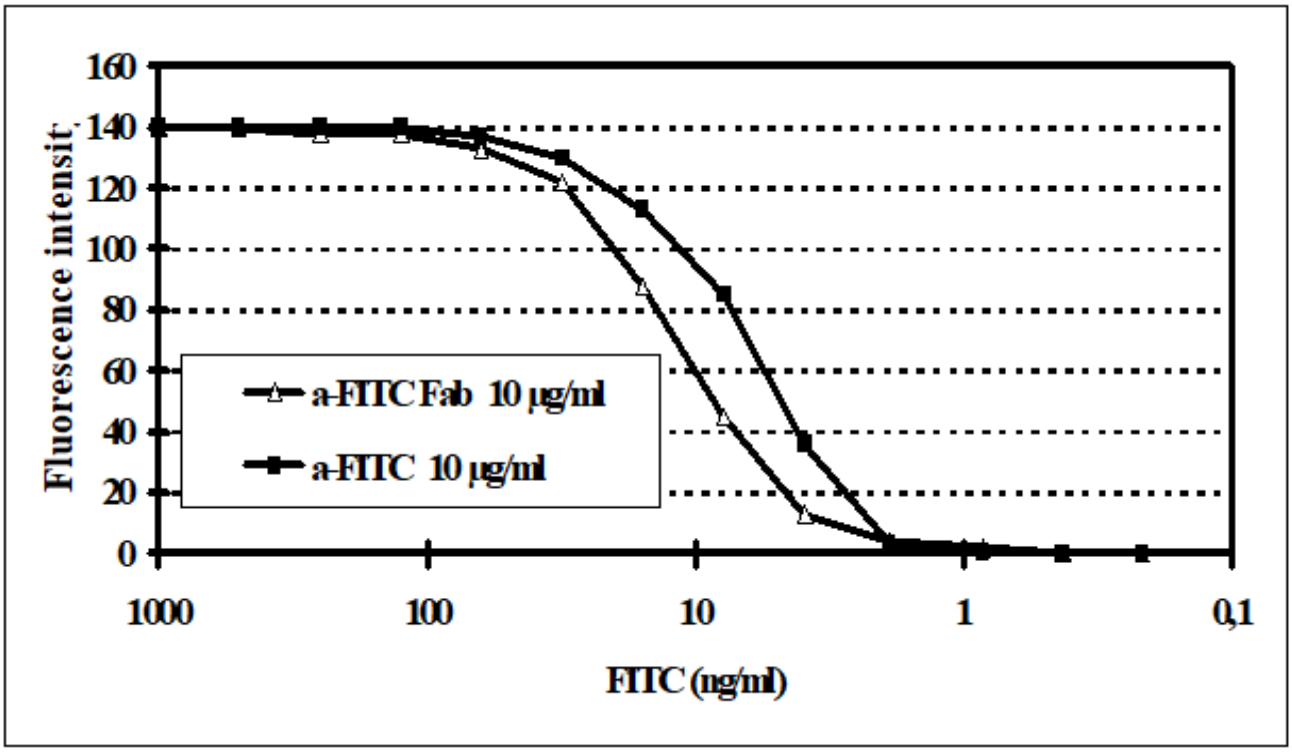

Figure 1. FITC fluorescence quenching by anti-FITC monoclonal antibody and its Fab fragment. Fluorescence intensity presented in relative units of the fluorimeter. Excitation and emission wavelengths were set to 493 and 512 nm. Measurements were taken at pH 7.2 at 21 °C. The figure demonstrates data of one typical measurement. Scattering between five independent assays was under 3%.

In the figure, we demonstrated two separate curves of fluorescence intensity – one for 10 µg/ml anti-FITC antibody and the other for 10 µg/ml anti-FITC Fab – against the concentration of FITC. It is striking that at 15 ng/ml FITC concentration, the fluorescence was quenched by 50% already in the case of anti-FITC Fab. The fluorescence was quenched by 50% in case of anti-FITC at around 5 ng/ml FITC concentration. At the equal paratope and hapten ratio (when the absolute number of hapten molecules theoretically equals the binding sites of the antibody molecules) , total (100%) quenching of fluorescence occurred. At a higher hapten concentration – over 200 ng/ml – a slight inner-filter effect can be seen at the beginning of the

curve. At about the threshold concentration of fluorophore (that is, when the optical density of the solution measured at its excitation peak equals 0.05), the partial quenching effect of a few anti-FITC molecules causes the same phenomenon as dilution. In spite of the lesser quantity of fluorescent compound actually emitting, the fluorescence intensity virtually enhances. Afterwards, with the addition of more antibody, the fluorescence intensity declines quickly to total quenching of fluorescence. This occurs at different hapten and paratope molecular ratios, depending on their total concentration, which is deductible from the law of mass action.

In the following phase of our study, we tried to analyze how this non-covalent molecular interaction – an immune bond – can modify the physico-chemical properties of an antigen molecule to such a fundamental degree.

In general, when the fluorescence intensity of a given fluorescent molecule decreases because of interaction with another molecule, the first interpretation that emerges is spectral shift. We investigated this possibility too, but found no remarkable spectral change if FITC molecules were bound chemically onto the surface of different protein carriers (Figure 2).

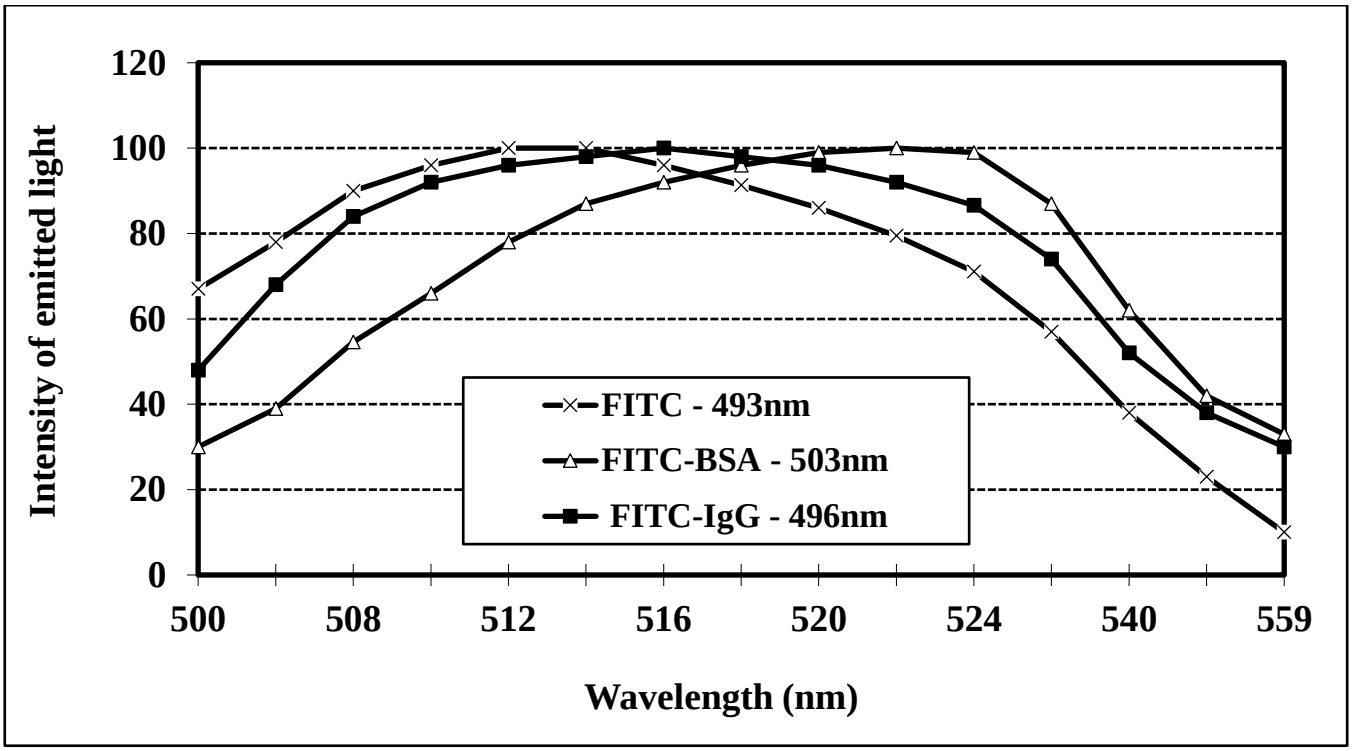

Figure 2. Emission spectrum of FITC covalently bound onto different protein molecules. Excitation wavelengths were set to 493 nm for free FITC, 503 nm for FITC-BSA, 496 nm for FITC-IgG1. Measurements were taken at pH 7.2 at 21 °C. Scattering between three independent measurements was under 5%.

Since covalent binding onto the surface of such big molecules as immunoglobulins or albumin (BSA) did not result in any spectral-shift bigger than 10 nm, it is hard to uphold the assumption that a simple, non-covalent molecular association between the small fluorophore and a relatively big partner molecule (a specific antibody in our model) causes fluorescence quenching. This result suggests that the mechanism of fluorescence quenching caused by anti-FITC monoclonal antibody is different from the covalent chemical binding, because it must be the unique nature of a specific immune bond that causes quenching.

To find a better explanation for this phenomenon, we compared the molecular movement and rotational freedom of free FITC

with FITC molecules covalently bonded to proteins, or with immunologically bonded FITC by anti-FITC or its Fab fragment using fluorescence polarization measurements. Results of these measurements showed further fundamental differences in the physico-chemical nature of covalent (chemical) and non-covalent (immunological) bonds. Using this technique, we were able to quantitatively detect the slightest changes in the rotation and/or flexibility of fluorophore molecules if they were limited in any way by any other molecule. The results of our fluorescent polarization measurements are presented in Figure 3.

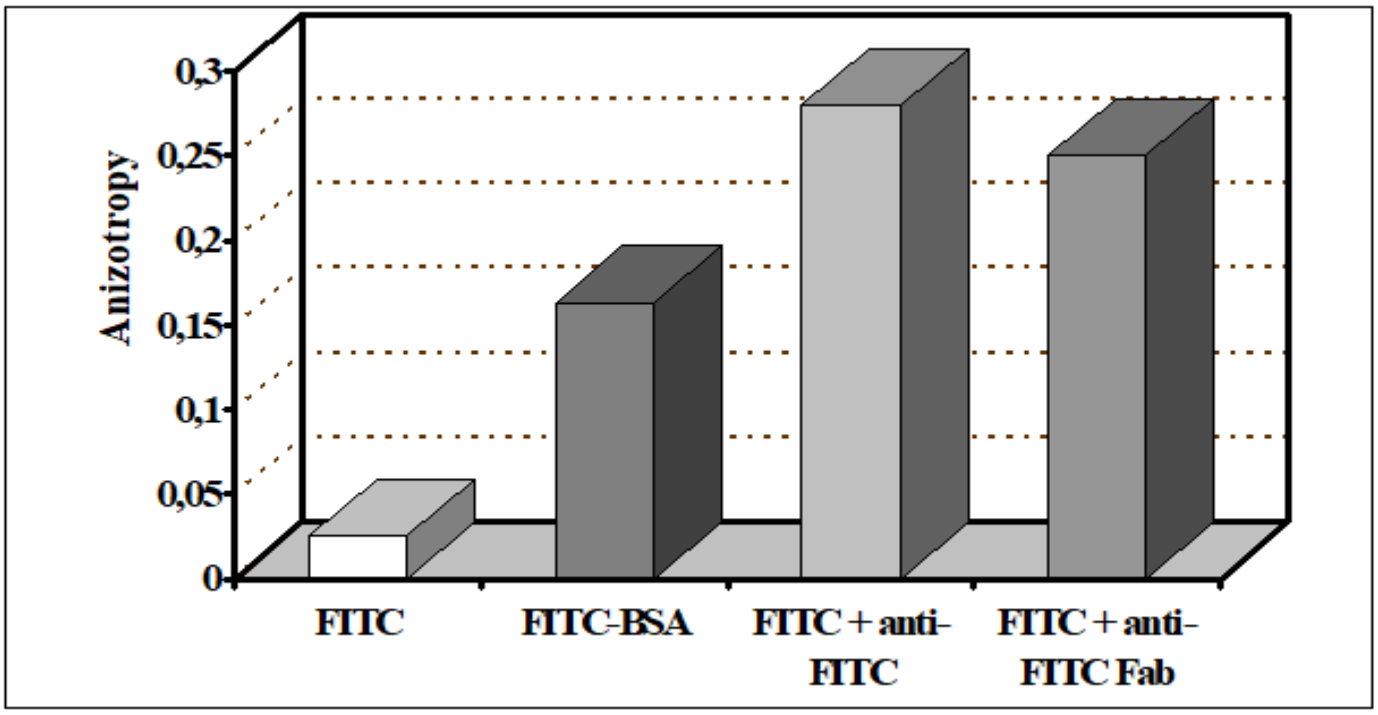

Figure 3. Fluorescence anisotropy of free FITC and FITC bound to proteins chemically or bound by anti-FITC and anti-FITC Fab. Excitation wavelengths were set to 493 nm for FITC, 503 nm for FITC-BSA, 496 nm for FITC-IgG1 and FITC-Fab, while the emission wavelengths were 512 nm for FITC, 522 nm for FITC-BSA, and 515 nm for FITC-IgG1 and FITC-Fab. Measurements were taken at pH 7.2 at 21 °C. The figure demonstrates summarized data of five independent measurements. Scattering was under 3%.

According to the Perrin equation, fluorescence anisotropy of a given fluorophore depends on its molecular microenvironment. This equation says that the bigger the anisotropy, the shorter fluorescence lifetime and the longer rotation correlation. In other words, an increase in anisotropy means that a fluorophore can transmit the energy it absorbs to another molecule in a very short time. This occurs because there is a very close association between the two molecules, which in some cases leads to instant channelling of absorbed energy from the fluorophore to the other molecule; moreover, this interaction can almost totally block the rotation of the little fluorescent molecule.

Based on this equation, we can draw basic conclusions concerning the molecular interactions demonstrated in Figure 3. The free movement and rotation of FITC molecules gets partially restricted after conjugation with higher molecular weight carrier proteins, such as BSA, or an indifferent, mouse IgG, characterized by increased anisotropy. However, the molecular movement of FITC is definitely inhibited after the incubation with specific anti-FITC monoclonal antibody. Immunological binding has a much more dramatic effect on FITC rotation and energy transfer than covalent chemical binding to indifferent proteins, including immunoglobulins with the same isotype (IgG1) the molecules have. The anisotropy was 100% higher in case of immunocomplexes formed by

FITC and anti-FITC (or anti-FITC Fab) than in case of covalent binding to the same isotype (IgG1) the molecule has. Anisotropy difference of only 5% was measured between the whole anti-FITC IgG1 and their Fab fragment during the formation of immunocomplexes with free FITC.

Another proof of a specific molecular movement-inhibiting, "rigidizing" effect of immune binding compared to that of covalent, chemical binding came from fluorescence lifetime measurements. These data are demonstrated in Figure 4.

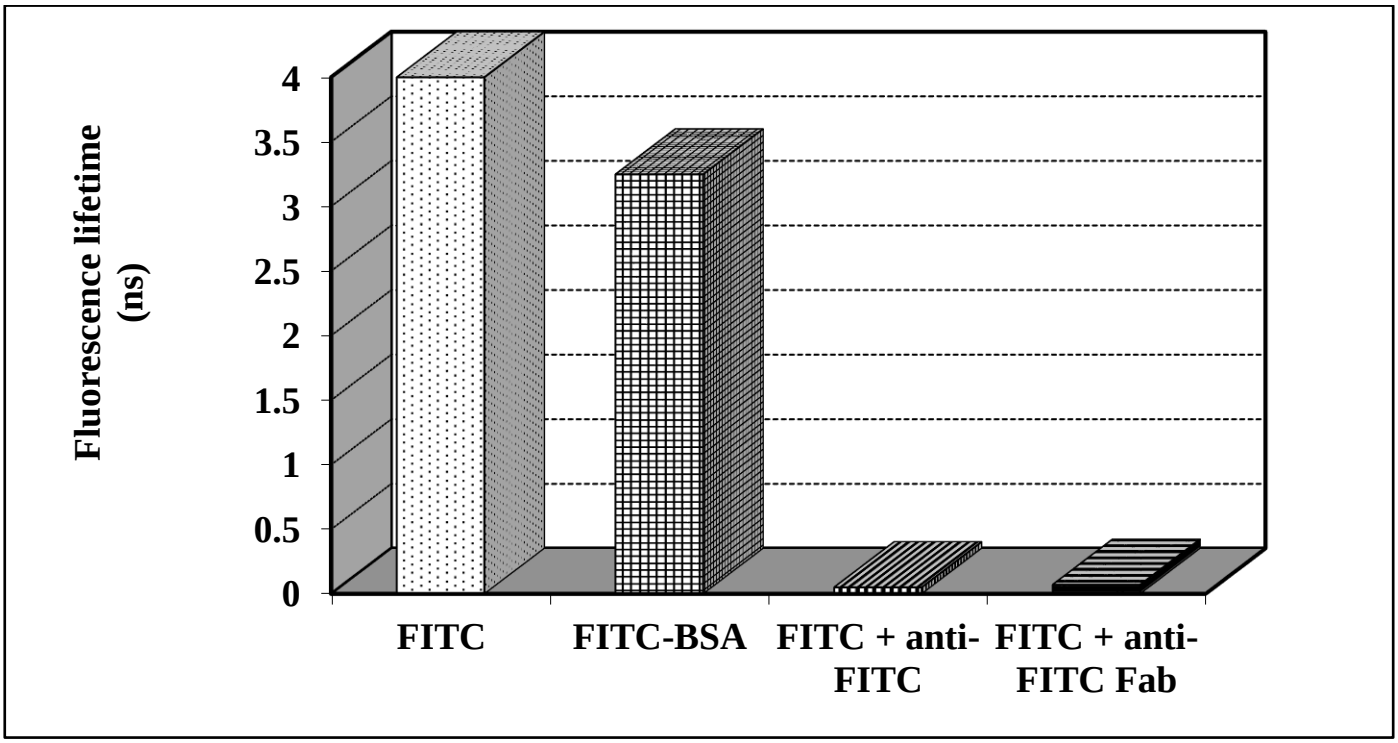

Figure 4. Fluorescence lifetime of free FITC and FITC bound to BSA, bound by anti-FITC IgG1 and anti-FITC Fab. Excitation wavelengths were set to 493 nm for FITC, 503 nm for FITC-BSA, 496 nm for FITC-IgG1 and FITC-Fab. Measurements were taken at pH 7.2 at 21 C. The figure demonstrates summarized data of five independent measurement. Scattering was under 3%.

Fluorescence lifetime gives us valuable information on the milieu surrounding the fluorophore molecule. The shorter the life spans, the quicker the energy transfer between the fluorescent molecule and its environment – that is, the interaction between the two molecules is stronger. It is very well demonstrated that lifetime is greatly affected by the milieu: FITC in PBS has a lifetime of about 4 ns, while BSA-coupled or IgG1-coupled FITC has a bit shorter lifetime, but on the same scale. We practically cannot measure the lifetime of FITC in FITC–anti-FITC (or FITC–anti-FITC Fab) complexes, which is not surprising, since there is practically no fluorescence signal because of quenching. The lifetime of FITC measured was about 4 ns when there was free unbound FITC in the reaction mixture. However, when anti-FITC immunoglobulins or their Fab fragments consumed all free FITC molecules, together they formed a so-called "dark complex". This meant there was no measurable fluorescence signal, so we could determine that lifetime equalled zero.

The "rigidization" of FITC molecules by immunobinding to anti-FITC IgG1, characterized by increased anisotropy and a shorter fluorescence lifetime, is a really striking phenomenon. However, this "high affinity interaction and energy transfer" theory simply cannot explain another astonishing finding that light absorption of FITC is similarly affected – inhibited – by anti-FITC as its fluorescence intensity. This is presented in Figure 5.

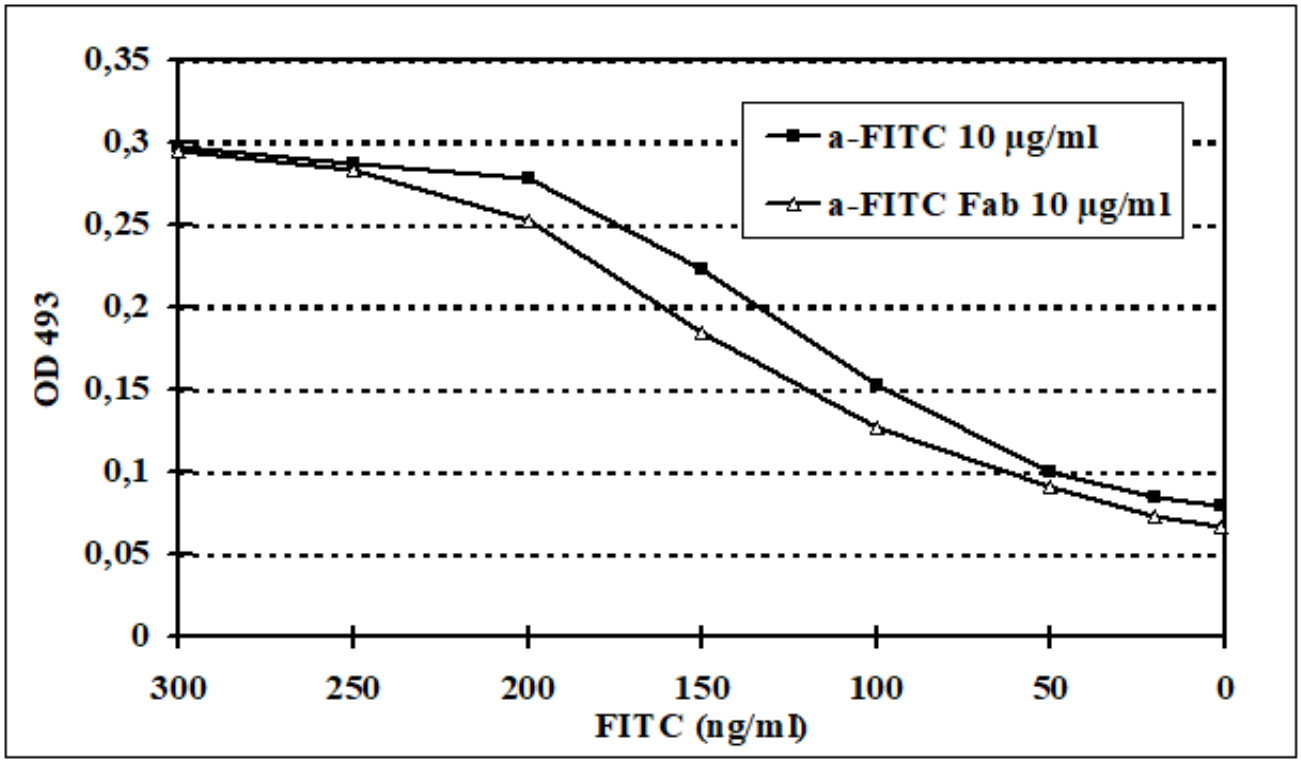

Figure 5. Light absorption of immunocomplexes formed by anti-FITC IgG1 and anti-FITC Fab plotted against the concentration of FITC. Excitation wavelengths were set to 493 nm. Measurements were taken at pH 7.2 at 21 C. Scattering between two independent measurements was under 2%.

Based on these data, it can be said that both fluorescence intensity and light absorption are greatly decreased by anti-FITC IgG1 or its Fab fragment. While the energy transfer from the excited fluorophore molecule can really be responsible for the decreased fluorescence intensity, it cannot explain light absorption at all. No remarkable differences were found between the whole immunoglobulin IgG1 (molecular weight is approximately 150 kD) and its Fab fragment (approximate molecular weight is 45 kD). We did not find any spectral shift caused by anti-FITC monoclonal IgG1 antibody or Fab fragments.

The difference between polar and non-polar microenvironment was analyzed. Figure 6 demonstrates the effect of acetone as a non-polar solvent on FITC fluorescence.

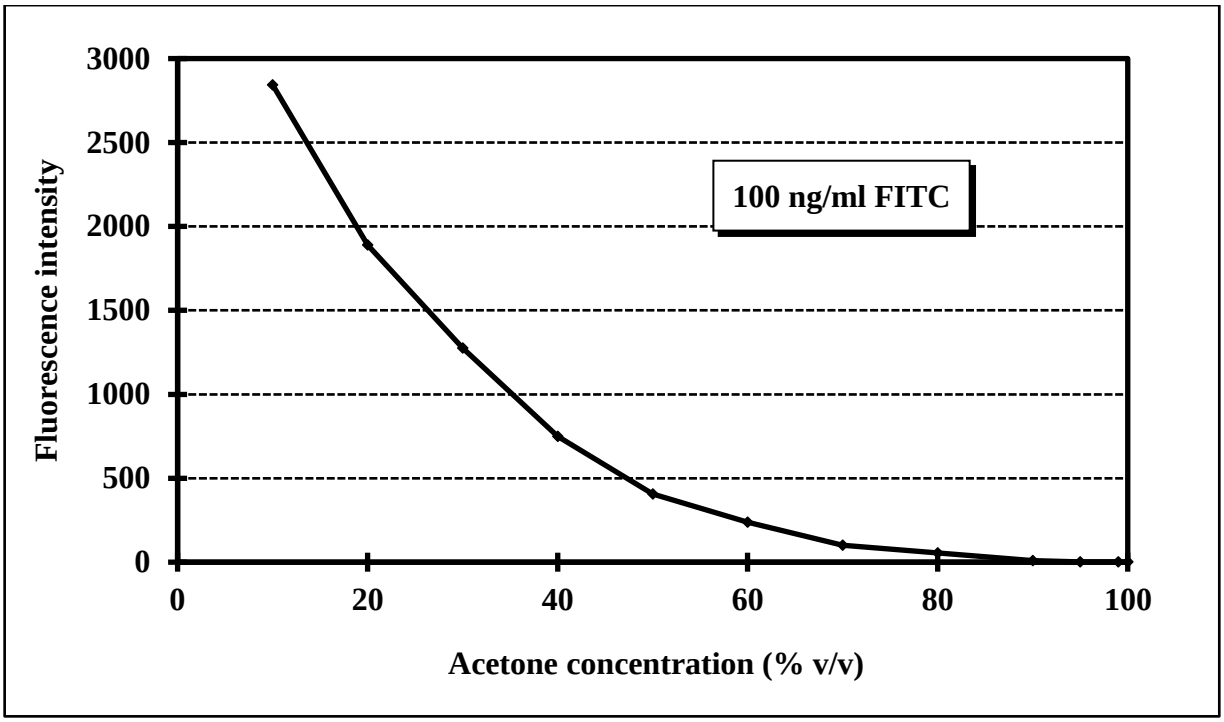

Figure 6. Fluorescence intensity of FITC dissolved in mixtures of acetone and water plotted against the concentration of acetone. Excitation and emission wavelengths were set to 493 and 512 nm, respectively. Fluorescence intensity presented in relative units of the fluorimeter. Measurements were taken at pH 7.2 at 21 C. The figure shows a typical curve. Scattering between three parallel measurements was under 2%.

The data clearly show that acetone has a similar decreasing effect on FITC fluorescence as immunobinding by anti-FITC IgG1 monoclonal antibody.

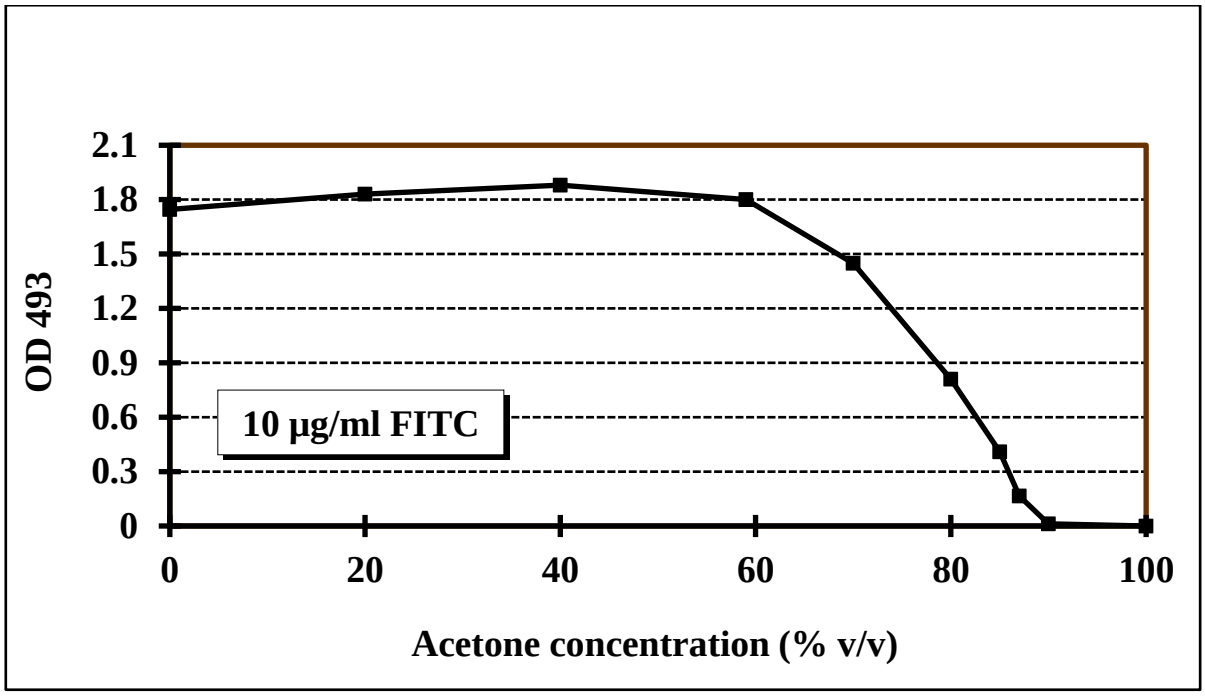

Figure 7. Light absorption of FITC in mixtures of acetone and PBS measured at 493 nm plotted against the concentration of acetone. Measurements were taken at pH 7.2 at 21 C. The figure shows a typical curve. Scattering between three parallel measurements was under 2%.

Table I. Spectrum assignation

FITC		
Position	**Intensity**	**Assignment**
682,303	56,020	C=C
728,317	72,842	=CH
761,780	67,621	=CH
855,877	43,726	=CH
1462,625	33,307	CC_{Ar}
1487,071	54,354	CC_{Ar}
1534,027	35,647	CC_{Ar}
1596,729	13,929	CC_{Ar}
1642,077	65,418	CO
2033,843	36,576	$_{as}N=C=S$
3052,083	80,938	=CH
3071,663	80,675	=CH
Anti-FITC (IgG1) FITC immunocomplex		
Position	**Intensity**	**Assignment**
1332,642	78,635	Amid III
1410,816	65,668	$_{s}CO_2^-$, zwitter-ion
1441,279	81,034	$C=C_{Ar}$*
1508,936	73,703	Amid II, $_{s}NH_3^+$
1632,279	41,895	Amid I, $_{as}NH_3^+$, NH_2 flodsheet
2037,691	86,476	NH_3^+
2964,388	79,044	Aliphatic CH
3069,372	71,81	OH, humidity
3426,762	62,718	OH, humidity
Anti-FITC (IgG1)		
Position	**Intensity**	**Assignment**
1333,148	76,502	Amid III
1411,488	62,640	$_{s}CO_2^-$, zwitter-ion
1508,429	69,217	Amid II, $_{s}NH_3^+$
1632,578	44,031	Amid I, $_{as}NH_3^+$, NH_2 flodsheet
2037,599	84,468	NH_3^+
2964,737	71,120	Aliphatic CH
3090,425	62,214	OH, humidity
3236,178	68,515	OH, humidity
Anti FITC (IgG1) FITC covalent binding (FITC labbeled IgG1)		
Position	**Intensity**	**Assignment**
1289,048	92,069	Amid III
1411,747	86,795	$_{s}CO_2^-$, zwitter-ion
1459,746	86,069	$C=C_{Ar}$*
1510,120	83,004	Amid II, $_{s}NH_3^+$
1534,883	83,579	$C=C_{Ar}$*
1648,909	82,896	Amid I, $_{as}NH_3^+$, NH_2, disorganized protein sequences
1656,884	82,505	Amid I, $_{as}NH_3^+$, NH_2 helix, CO*
2036,716	89,831	NH_3^+
2933,257	96,469	Aliphatic CH
3355,729	93,908	OH, OH, humidity

*FITC

Fluorescence quenching increases proportionally to the concentration of acetone and total (100 %) quenching of fluorescence occurs at 96 % acetone concentration (v/v), when FITC molecules practically have no hydrate wrap. Moreover, the same phenomenon can be observed with respect to light absorption (Figure 7).

Infrared spectroscopy can analyze more precisely the molecular connections between the antigen and antibody structures, including the influence of the microenvironment. According to our Fourier-transform infrared (FT-IR) spectroscopic measurements in case of covalent binding peak 2033 cm-1 of FITC's N-C-S group (which is the covalent binding region) showed remarkable changes; it disappeared. Only the peak 2036 cm-1 of anti-FITC's NH_3^+ groups was present. Figure 8 demonstrates the FT-IR spectrum of free FITC.

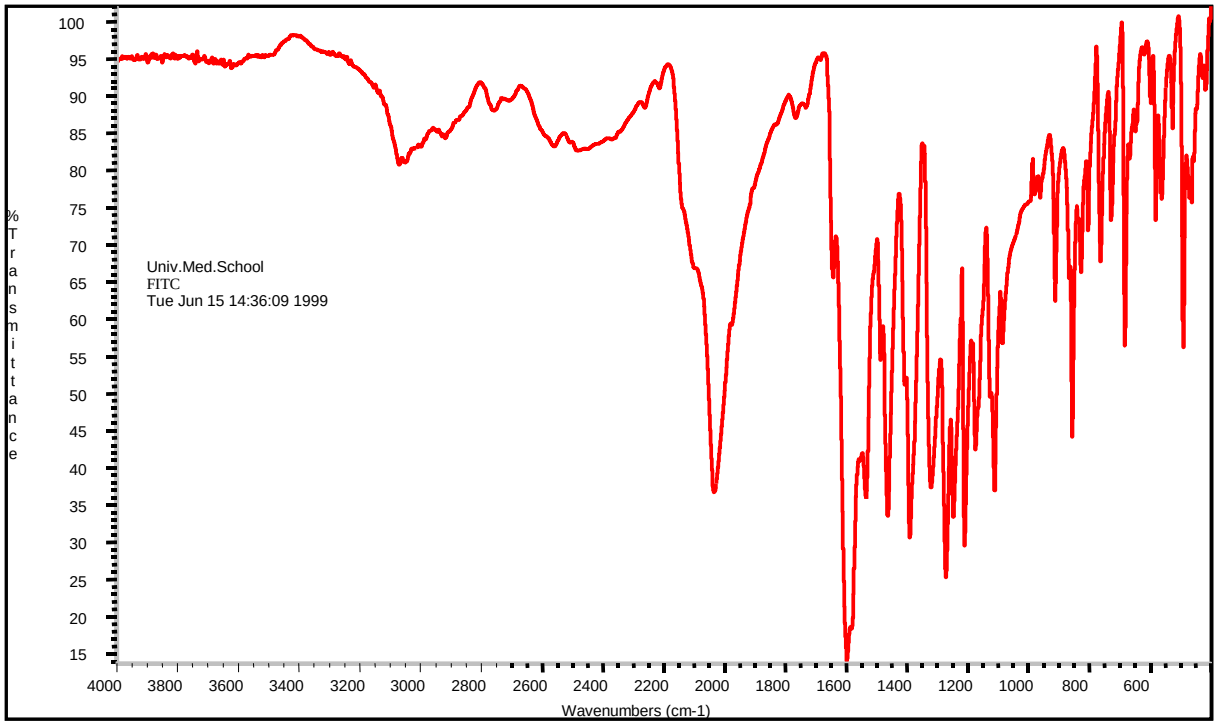

Figure 8. Fourier-transform infrared spectra of free FITC molecules. The 0.2 mg sample was pastillized in KBr at a range of 400-4000 cm-1 and studied with a 4 cm-1 resolution. The details of peak assignations are presented in Table I.

Figure 9 shows the spectrum of free anti-FITC IgG1, and Figure 10 shows the spectrum of FITC covalently bound to monoclonal anti-FITC IgG1.

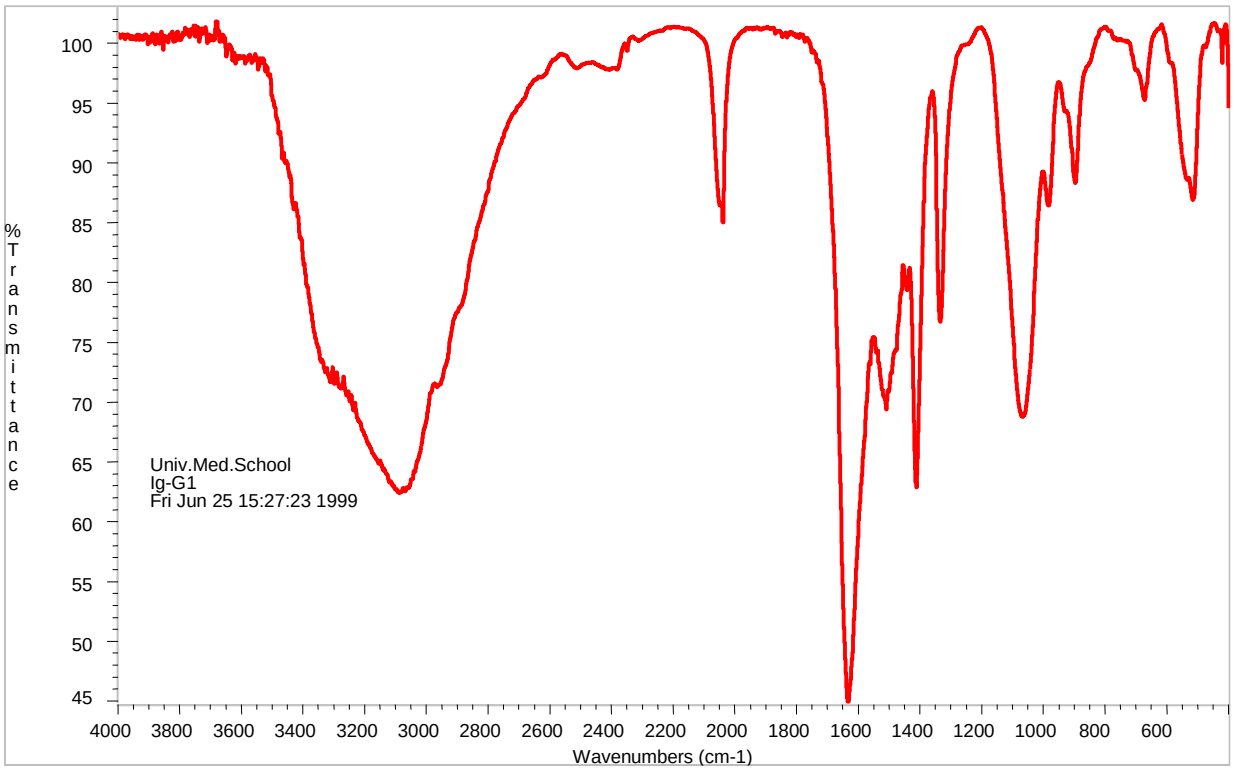

Figure 9. Fourier-transform infrared spectra of anti-FITC IgG1 monoclonal antibody molecules. The 0.5 mg sample was pastillized in KBr at a range of 400-4000 cm-1 and studied with a 4 cm-1 resolution. The details of peak assignations are presented in Table I.

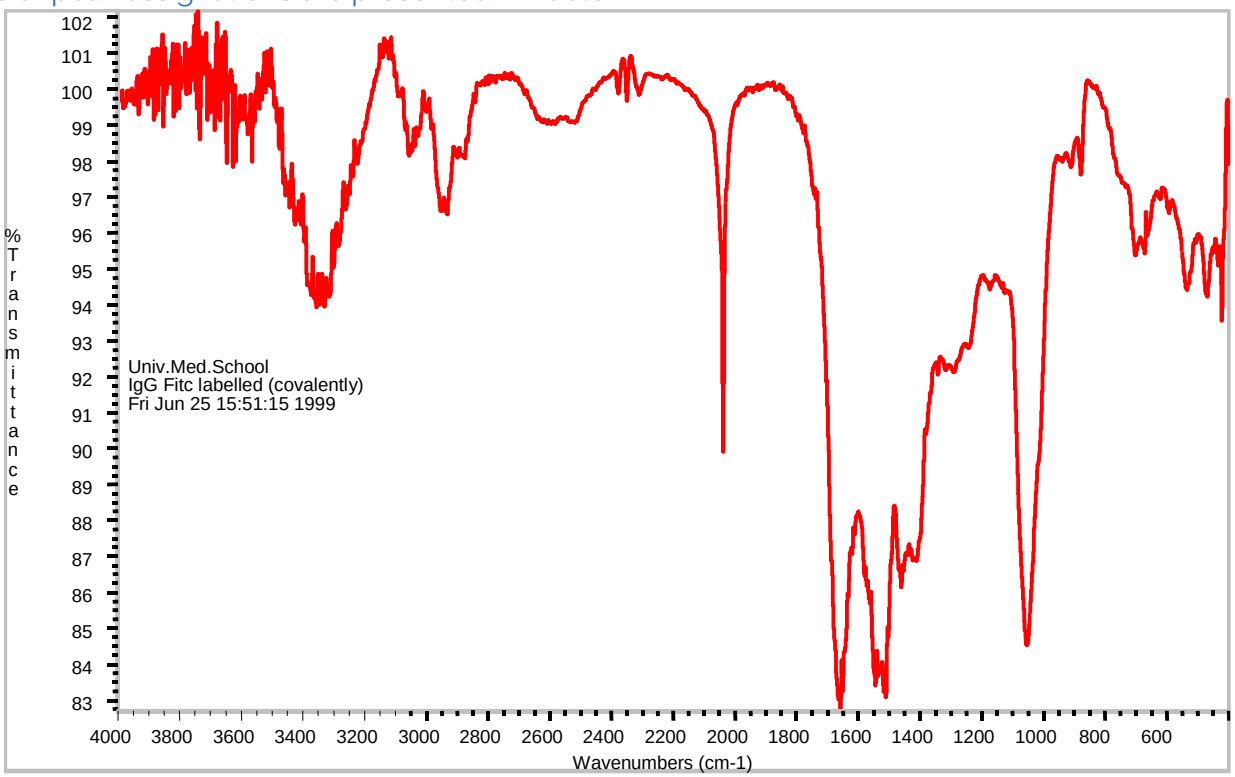

Figure 10. Fourier-transform infrared spectra of IgG1 monoclonal antibody covalently labelled by FITC. The 0.5 mg sample was pastillized in KBr at a range of 400-4000 cm-1 and studied with a 4 cm-1 resolution. The details of peak assignations are presented in Table I. Major characteristics of native FITC and IgG1 molecules can be found in the spectrum of the complex.

Specific binding sites of the anti-FITC IgG1 were blocked before and during the covalent FITC-labelling. The spectrum contains characteristic peaks of both FITC and anti-FITC IgG1. The peaks of poly-aromatic cyclical structures (responsible for fluorescence of FITC molecules) added to the peaks of the anti-FITC IgG1 molecule: peaks 1459 cm-1 and 1534 cm-1 were clearly shown and peak 1642 cm-1 shifted and added to anti-FITC's 1656 cm-1 peak. In case of covalent binding, we did not find hydrophobic interactions between the antigen and antibody molecules: the anti-FITC IgG1's 3236 cm-1 OH peak shifted to 3355 cm-1. In case of immunobinding between the FITC and anti-FITC IgG1, the antigen's N-C-S group was assigned to peak 2033 cm-1, that is, the covalent binding region of FITC. The peak 2033 cm-1 showed no remarkable changes during antigen-antibody reactions, but only shifted and added to the peak 2037 cm-1 of NH_3^+ groups on antibodies. The polyaromatic cyclical structure, which is responsible for fluorescence, showed significant physico-chemical changes: peaks from 1462 cm-1 to 1642 cm-1 disappeared and/or shifted to 1441 cm-1. However, hydrophobic interactions were detected between the antigen and antibody molecules: the antibody's 3090 cm-1 and 3236 cm-1 peaks of OH groups shifted to 3069 cm-1 and 3426 cm-1, and their transmittance decreased (Figure 11).

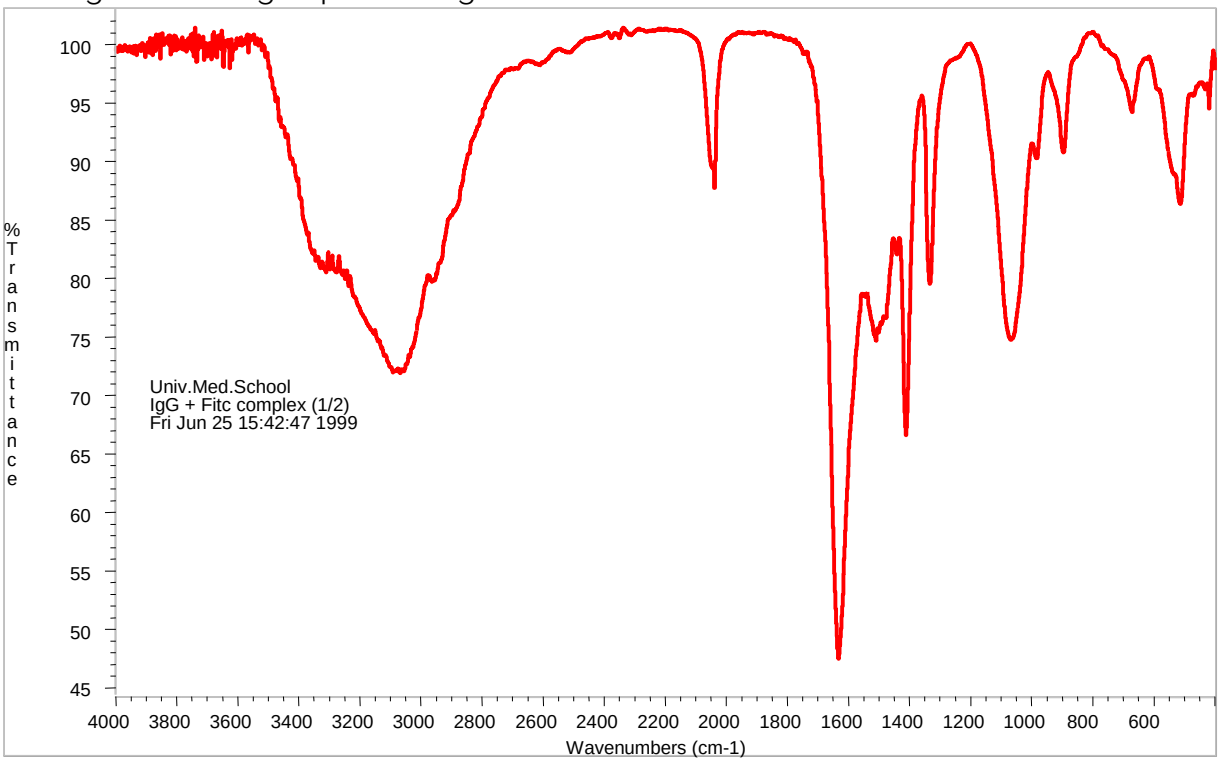

Figure 11. Fourier-transform infrared spectra of immunocomplexes formed by FITC and anti-FITC IgG1. The 0.2 mg sample was pastillized in KBr at a range of 400-4000 cm-1 and studied with a 4 cm-1 resolution. The details of peak assignations are presented in Table I. No polyaromatic peaks of FITC were found (1400-1600 cm-1) and peaks of OH groups of free anti-FITC IgG1 (3000-3400 cm-1) changed remarkably in the immunocomplex.

Discussion

Our conclusion is that immunobinding itself can modify the electron cloud of antigens so dramatically that they could not exhibit the same electrostatic structures anymore. We concluded that this extreme structural modification results from making it impossible for the FITC molecule to get in contact with water. It has been known for a long time that solvents have a basic influence on the fluorescence of fluorescent compounds (30, 31). Considering this, we suspected that this phenomenon is caused by

the lack of water in the microenvironment of FITC bound by anti-FITC IgG1. We tried to model water exclusion by using non-polar solvents for making our samples. The spectral shifts and fluorescence quenching in different acetone (organic solvent) and water mixtures reflect a general effect on the hydrogen-bonding environment of the FITC through water-acetone hydrogen bridges and specific effects due to direct FITC -acetone hydrogen bonding. By increasing acetone concentrations, the FITC-water connections change to FITC-acetone interactions, resulting in basic modifications of hydrogen bonds due to fluorescence quenching. Both the absorption spectra and fluorescence properties (quenching) appear to be dominated by increasing hydrophobicity, indicating that the spectral shifts of the FITC can be used as an indicator of its hydrogen-bonding environment. Application of fluorescein as a probe of hydrogen bonding in the microenvironment immediately surrounding the fluorophore illustrated the effect with reference to the fluorescein-anti-fluorescein antibody complex (30). This finding fits well in our results (as demonstrated in Figure 2, Figure 6, and Figure 7), where it appears that binding sites of our anti-FITC monoclonal antibody and the recognized antigen (FITC) are increasingly dehydrated and become hydrophobic during the immunocomplex formation.

Hydrophobic interactions between the antigen and antibody molecules seem to be essential in the physico-chemical event of antigen-antibody reaction, which is supported both by our fluorescent spectroscopic and fluorescent polarization measurements and other data from the relevant literature (15, 23, 30, 31). Our model experiment, involving non-polar solvent and water mixtures, supported our hypothesis about the importance of hydrophobic interactions in the stabilization of the antigen-antibody ligands. However, further experimental evidence is needed.

The results of the infrared spectroscopic analysis can prove the hydrophobic nature of immunobinding between anti-FITC and FITC. This hydrophobic interaction also perturbed the electron cloud of the polyaromatic cyclical structure. These results can also prove the quenching effect caused by anti-FITC monoclonal antibody.

Our fluorescent and infrared spectroscopic data suggest that the hydrophobic connections not only participate in the stabilization of antigen-antibody complexes, but also modify the physico-chemical nature of participating molecules.

The main ligand-induced conformational changes on the immunoglobulin molecules causing a basic modification of their functions are well-known. However, the same physico-chemical influences on the fine structure of antigens have been less analyzed so far due to technical difficulties (9, 10, 11).

In the original FITC – anti-FITC model, the fluorescence-quenching effect was observed during antigen-antibody reactions (9, 32). The fluorescent activity of FITC molecules as antigens in this model system seems to be beneficial for studying fine structural changes in the antigen after binding with a specific immunoglobulin (9, 33).

Using fluorochrome-labelled anti-idiotypic antibodies and fluorescent spectroscopic measurements, experimental data published suggest the importance of water molecules in the ligand formation (2). Our data, together with previous findings, help understand the molecular dynamics of immunobinding (2, 3, 7). The immunocomplex formed by hydrophobic regions of a specific epitope on the antigen and the recognition part on the antibody molecule stabilized with the surrounding hydrate shell seems to be thermodynamically strong enough for stable molecular binding without a covalent chemical interaction (34, 35).

In our model, direct observations were possible for the physico-chemical changes on the bound antigen because the quenching effect of FITC fluorescence during the immunocomplex formation was analyzed by different and relevant spectroscopic techniques. Our pre-sent fluorescent polarization and infrared spectroscopic measurements reflect a local decrease in the hydration degree in the

submolecular area of a specific ligand between the small antigen (hapten) molecule and the hypervariable region of IgG. This "exclusion" of water molecules from the binding surfaces occurs due to a dramatic modification of the molecular microenvironment. Changes in hydration can influence the functions of the immunoglobulins and the physico-chemical appearance of the antigen at the same time (36). Participation of antigens in immunocomplex formation can cause altered immunoreactivity in further physiological and pathological immune reactions.

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Disclosure

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[1] **Author contribution.** single author