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ORIGINAL ARTICLE

Antigen-Induced Activation of Antibody Measured by Fluorescence Enhancement of FITC Label at Fc

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Abstract Three anti-carbohydrate antibodies of defined specificity isolated from plasma were used to demonstrate that macromolecular antigen binding caused considerable enhancement of fluorescence of FITC-labeled antibody. Mono and disaccharide antigens which could compete with the large antigens in antibody binding could not however produce any increase in fluorescence. Fluorescence enhancement in a given antibody sample increased with the size of the occupying macromolecular antigen. Conversely in antibody samples of same ligand specificity isolated from plasma of different individuals, fluorescence enhancement produced by the same antigen correlated with specific activity of the antibody sample. Removal of Fc part of antibody, confirmed by electrophoresis and Fc-specific antibody binding, caused abolition of most of the antigen-driven fluorescence increase. Since antigen binding sites of antibodies were protected during FITC labeling, the above results suggest that conformational shift in Fc produced by occupation of binding sites by large antigens resulted in the enhancement of fluorescence of FITC tags on Fc. Data provides a tool for detection and measurement of specific ligands using fluorolabeled whole antibodies.

Keywords Anti-carbohydrate antibody · FITC · Fab · Fc · Fluorescence enhancement

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Introduction

Consequent to antigen binding at the hypervariable regions of their antigen-binding (Fab) chains antibodies are activated to mediate processes such as complement fixation, mast cell activation and triggering lymphocytes towards differentiation or tolerance. The latter function is attributed to conformational shift brought about in the constant and crystallizable (Fc) part in response to antigen binding [1]. Structural studies have shown that binding of antigen induces conformational changes in the antibody, antigen or both [2, 3]. Conformational changes in the binding site of the antibody has been proposed to result from an induced fit brought about by the antigen [4] leading to alteration in size, shape and charge distribution in the antigen binding pocket [5]. Antigen binding has been suggested to trigger a signal which is transmitted from the variable (Fv) region to the Fc in the form of conformational changes in the Fc portion [6–8]. In support to this hypothesis smaller antigens capable of producing only local and limited conformational changes in the antigen binding site do not cause any change in the Fc part nor activate any effector function of the antibody [7]. The mechanism of transmission of signal from Fab to Fc upon antigen binding is not yet clear though it has been assumed that inter-chain disulfide bond plays a major part.

Abe et al. [9] reported a significant antigen-dependent fluorescence enhancement when fluorolabeled single chain variable region of antibody was used. In the present study we demonstrate using naturally occurring human plasma anti-carbohydrate antibodies that fluorescein isothiocyanate (FITC)-labeled whole antibody shows significant increase in fluorescence upon antigen binding and that conformational changes in Fc part are largely responsible for this effect. We also show that increase in fluorescence of FITC-labeled antibody is proportional to a) affinity of antigen when various

antigens bind to a given antibody sample and b) specific activity of the antibody when a given antigen binds to antibodies of varying specific activity isolated from different individuals. Increase in fluorescence can be used as a measure of antigen binding affinity and to detect the presence of specific ligands. Naturally occurring anti-carbohydrate antibodies were used for the study since these are easily prepared in pure form and antigens and their analogues are readily available.

Materials and Methods

Materials

Soybean trypsin inhibitor, bovine thyroglobulin (Tg), orthophenylenediamine (OPD), horse radish peroxidase (HRP), hydrogen peroxide, 1-O-methyl-α-D-glucopyranoside, cellobiose, melibiose, sulpho-NHS-biotin, avidin-HRP, Tween-20, sodium cyanoborohydride, FITC, papain, iodoacetamide, Biogel P-4, dextran (15–400 kDa), soluble guar gum and streptavidin were purchased from Sigma-Aldrich, Bangalore, India. Polystyrene 96-well microplates (MAXISORP) were purchased from Nunc, Roskilde, Denmark. Polystyrene 96-well microplates used for fluorescence measurement was from Dynex Technologies, USA. Antibodies to human IgA, IgM and IgG raised in rabbit were obtained from Dako, Denmark. Sephadex G-200 was from Pharmacia Fine Chemicals, Uppsala, Sweden.

Isolation of Antibodies

Three human plasma carbohydrate specific antibodies viz a) anti-α-galactoside antibody (anti-Gal) unique to humans and other primates and specific to terminal α-linked galactose moieties, b) dextran- binding immunoglobulins (DIg) recognizing dextran and other α- glucosides and c) anti-β-glucan antibody (ABG) specific to β-glucosides and other polymers were isolated from out-dated plasma donated by healthy volunteers (18–40 years) and supplied by the Department of Transfusion Sciences of this institute in accordance with Institutional Ethics Committee (IEC) clearance (511/2013). Since a section of circulating anti-Gal had been observed to form immune complex with lipoprotein (a), plasma anti-Gal was prepared by affinity chromatography on cross-linked guar galactomannan gel after dissociating the lipoprotein using specific sugar [10]. Briefly plasma (70 ml) treated with 200 mM galactose overnight was subjected to ultracentrifugation at 202,000 g in 4.96 density at 4 °C in 4 ml tubes. Bottom 30 % volume from tubes were pooled, dialysed against 20 mM potassium phosphate buffer containing 150 mM NaCl, pH 7.4 (PBS) and anti-Gal isolated by affinity chromatography as described earlier [11]. Matrices used for affinity chromatographic isolation of DIg and ABG were: Sephadex G-200 [12], and cellulose-celite column in 1:1 ratio [13].

FITC Conjugation of Antibodies

FITC coupling to antibodies was done essentially as described by Hudson and Hay [14]. Antibody samples were concentrated to 1 mg/ml, dialysed against 0.25 M carbonate-bicarbonate buffer (pH 9.0) and pre-incubated for 2 h with respective specific sugars (25 mM) to protect the antigen binding site from labeling. FITC (0.15 mg per mg protein) was then added and mixture kept overnight at 4 °C. Gel filtration using 25 ml Biogel-P4 column in PBS medium was used to separate the FITC labeled antibody from reagents.

Effect of Specific Ligands on Fluorescence of FITC–Labeled Antibody

FITC-labeled antibody (6.25 μg protein in 25 μl PBS) preincubated with and without specific disaccharide (25 mM) was mixed with 15 μg of candidate antigen in 15 μl PBS and incubated overnight at 4 °C. After diluting to 300 μl with PBS, fluorescence was measured in BIOTEK fluorescence reader using excitation at 485 nm and emission at 520 nm.

Determination of Specific Activity of Antibody

Specific activity of anti-Gal was defined as the ratio of thyroglobulin binding activity to immunoglobulin content of the same amount of antibody. These parameters were measured by enzyme-linked immunosorbent assay (ELISA). Polystyrene microwells (NUNC Immuno Break-apart, MAXISORB) were coated with thyroglobulin by 3 h incubation at 37 °C with the protein (2 μg in 200 μl PBS). Wells were washed with PBS containing 0.05 % Tween-20 (PBS-T) and blocked with PBS containing 0.5 % Tween-20 at 37 °C for 30 min. After washing again with 0.05 % PBS-T, wells were incubated with 50 ng anti-Gal in 200 μl 0.05 % PBS-T for 2 h at 4 °C, washed thrice with PBS-T (0.05 %) and bound antibody assayed by probing with a mixture of HRP conjugates of anti-human IgG, IgM and IgA (all 1.5 μg antibodies per ml) in PBS-T (0.05 %) and bound HRP activity was assayed by incubating for 15 min with 200 μl OPD (5 mg/ml) in citrate phosphate buffer, pH 5.0 containing 0.03 % H_2O_2, stopping the reaction with 50 μl of 12.5 % H_2SO_4 and reading the plates at 490 nm in ELISA reader (BIOTEK USA). To assay immunoglobulin content of anti-Gal 50 ng antibody protein directly coated on microwells as above were probed with the mixture of HRP conjugates of anti-human IgG, IgM and IgA used above and bound HRP activity assayed. The ratio of responses (OD at 490 nm) in the above two ELISA was taken as specific activity of the antibody.

Isolation of Fab from Papain Digested FITC- Labeled ABG

Papain digestion was done essentially by the method by Hudson and Hay [14]. Fifty μl each of 1 M cysteine and 20 mM EDTA along with papain (25 μg in 25 μl) was added to 1 mg/ml antibody (ABG) in 1 ml 100 mM sodium acetate-acetic acid buffer (pH 5.5) and mixture incubated at 37 ^{0} C for 6 h. Iodoacetamide (13.8 mg) was added and incubation continued for 30 min to stop the reaction and mixture dialysed against PBS. The digested mixture containing Fab and Fc were then passed through a 20 ml of cellulose-celite column (1:1) and washed with PBS. The bound Fab was then eluted by using 250 mM dextrose and dialysed against PBS.

Total Immunoglobulins from Plasma

From 10 ml plasma immune complexes and particulate matter were precipitated by 20 % ammonium sulphate and removed by centrifugation at 17, 418 g for 30 min. Ammonium sulphate concentration in supernatant was raised to 45 % and the precipitate containing mostly immunoglobulins was collected by a similar centrifugation as above and dialysed against PBS.

Other Preparations

An insoluble affinity gel, cross linked guargum (CLGG) was prepared by cross linking soluble guar gum (from *Cyamopsis tetragonolobus* beans) using epichlorohydrin employing a procedure described by Appukuttan et al. [15]. Melibiose and cellobiose covalently coupled to the non-glycosylated protein soybean trypsin inhibitor and termed TiM and TiC respectively were prepared by reductive amination using sodium cyanoborohydride [16]. Periodate activation of HRP and covalent conjugation of proteins to the enzyme was performed as described by Hyderman et al. [17] using protein and HRP in the ratio of 3:2 by mass. Proteins were estimated with bovine serum albumin as standard according to Bradford [18]. The total neutral sugar was estimated by phenol-sulphuric acid method of Dubois et al. [19] in a total volume of 5.5 ml with galactose as standard. Albumin from normal human plasma was isolated by affinity chromatography on blue-Sepharose as described by Travis et al. [20].

Statistical Analysis

Statistical analysis was done by Microsoft Excel 2000 version, (Redmond, WA, USA) and by Graphpad Prism 5. Comparison between groups was made using Student's *t test*, *P* value of<0.05 was considered significant.

Results and Discussion

Fluorescence Enhancement of FITC Labeled Antibodies upon Antigen Binding

Fluorescence of each of three antibodies anti-Gal, DIg and ABG, labeled with the fluorescent ligand FITC (Fig. 1a) was enhanced upon binding of corresponding macromolecular antigen, viz bovine thyroglobulin, dextran 150 kDa and TiC respectively (Fig. 1b), percentage increase being 26, 34 and 32 % respectively. Further, enhancement of fluorescence following binding of large antigens to each of the above antibodies was fully abolished by the presence of respective mono- or disaccharide antigens at 25 mM concentration. Trypsin inhibitor (Ti) or human plasma albumin used as control proteins was ineffective in causing any change in fluorescence of FITC-labeled antibodies (data not shown), indicating that it takes a specific ligand as part of a macromolecule to cause fluorescence enhancement in antibodies. FITC labeling of antibodies did not cause reduction in antigen binding activity of the antibodies or in their recognition by Fc-specific secondary antibodies (data not shown) owing primarily to protection of the binding site by small antigenic ligands during conjugation with FITC.

Total immunoglobulin fraction precipitated by 45 % ammonium sulphate saturation of plasma was also coupled with FITC to check whether the increase in fluorescence observed was due to any colligative effect regardless of antigen specificity. No increase in fluorescence was produced by ligands like thyroglobulin, TiM or TiC on FITC-labeled total immunoglobulin (data not shown).

The change in fluorescence could be due to conformational changes produced in the antibody upon antigen binding [1]. The effector function of an antibody is activated by conformational changes in CH2 domain of the Fc part [3, 21] which in turn is located around 80–100 A^0 away from the antigen binding site in the case of IgG [22] and much farther away in the case of IgM which assumes a staple conformation upon antigen binding [2]. Abe et al., [9] has concluded that the increase in fluorescence of fluorolabeled single chain variable region upon antigen binding is due to antigen dependent removal of quenching effect caused by the proximity of tryptophan residues in the antigen binding pocket. Contrary to this Schlessinger et al. [1] has shown that the change in circularly polarized luminescence (CPL) spectrum of antibody upon antigen binding was reversed by the removal of Fc by papain digestion. Most of the conformational changes in the antibody molecule take place away from the antigen binding site [21]. Antigen binding has been shown to profoundly change the conformation of the Fc part of antibodies enabling it to fix complement as well as trigger effector functions in other cells [1]. These reports along with the fact that binding site at the variable region were protected from fluorescence labeling

indicated that fluorescence increase caused by macromolecular ligands on FITC-labeled antibodies was mainly due to conformational changes in Fc part. Reversal of fluorescence shift by prior incubation of antibody with specific sugar confirmed that binding of macromolecular ligands was responsible for the observed fluorescence increase.

Fluorescence Enhancement in FITC-Labeled Antibody Increases with Size of the Macromolecular Antigen

DIg was used to study the effect of molecular size of antigen on fluorescence enhancement in FITC-labeled antibody since macromolecular ligands for this antibody (dextrans) were available in different molecular sizes ranging from 15 to 400 kDa. DIg is a naturally occurring anti-carbohydrate antibody with specificity for α-linked glucosides [23] which are also the monomers of dextran. Same amount (15 μg neutral sugar) of different varieties of dextran with molecular masses of 15 kDa, 34–40 kDA, 150 kDa and 400 kDa produced fluorescence increase of 17.8, 25.8, 32.9 and 34.9 % respectively in FITC-labeled DIg (Fig. 2). Variations in binding-induced Fc activation produced in DIg by the same number of dextran molecules differing in size are several times more than indicated by Fig. 2 since higher molecular weight dextrans are less numerous in a given mass. Results in Fig. 1b and 2 indicate that though monosaccharide moiety may be the basic sugar unit for anchoring of an antigen on a carbohydrate-binding antibody such as DIg, the larger antigens, unlike small sugars, engage in secondary interaction with regions in the antibody neighboring the binding site. Secondary interactions are likely to produce in the Fab region an induced fit akin to that produced by a substrate on an enzyme to an extent sufficient to produce a measurable conformational shift in the Fc region as well. Increase in fluorescence enhancement of FITC- antibody with increase in antigen size underlines this assumption. Our results agree with the findings of Oda et al. [7] that binding of small antigens are capable of producing only local changes in the binding site without activating effector functions. Further, Sela Culang et al. [21] has also shown that conformational changes in antibody takes place in the elbow angle between variable and constant domain as well as in a loop in the heavy chain constant domain and that these changes are significantly larger on binding of large antigens rather than binding of small antigens.

Fluorescence Enhancement in Antigen-Bound Antibody is a Measure of Antigen Binding Affinity

Anti-Gal that constitutes nearly 1 % of circulating IgG is specific to α-linked terminal galactosides and is synthesized only by higher primates such as man, apes and old world monkeys [24]. Using anti-Gal samples isolated from plasma of six individuals and varying in specific activity from 0.326 to 1.1

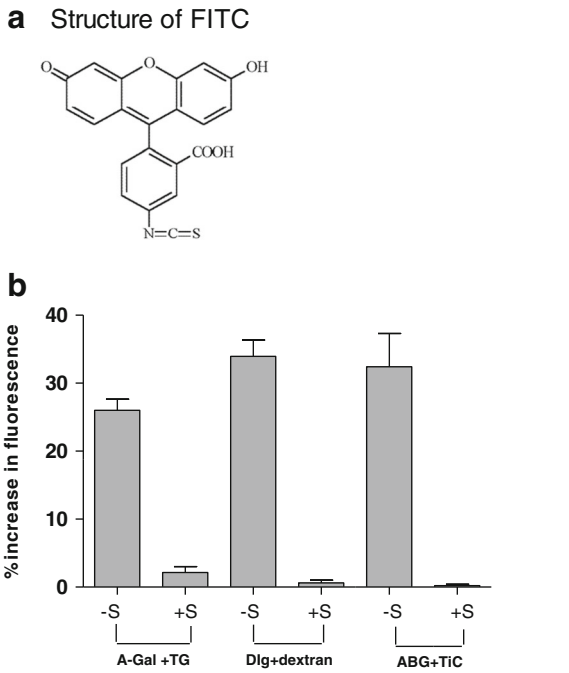

Fig. 1 **a** Structure of FITC; **b** Increase in fluorescence of antibodies in presence of specific macromolecular antigen: Antibody (6.25 μg) was mixed with 15 μg of respective macromolecular antigen overnight before measuring fluorescence (details under methods). Values are mean ± SD of 7 anti-Gal samples, 5 DIg samples and 5 ABG samples. Percentage increase over that of free antibodies shown. Antibodies pre-incubated with specific sugars (S: 25 mM melibiose for anti-Gal, 25 mM 1-O-methyl-α-D-glucoside for DIg and 25 mM cellobiose for ABG) served as control. TG: bovine thyroglobulin; TiC: cellobiose coupled to soybean trypsin inhibitor

(Fig. 3) it was observed that increase in fluorescence enhancement was proportional to the specific activity of the antibody when the same amount of FITC-anti-Gal and antigen (thyroglobulin) were used in all cases (Fig. 3). Apart from confirming antigen occupation at binding site as cause of fluorescence increase the results suggested that fluorescence

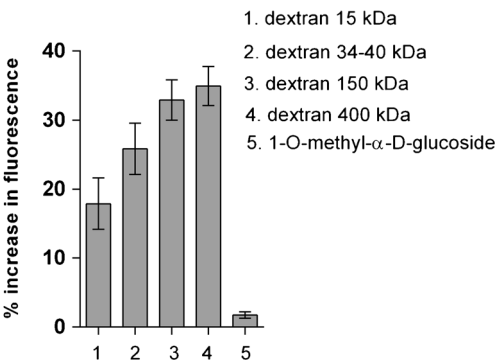

Fig. 2 Percentage increase in fluorescence of FITC-labeled DIg with dextran of different molecular weight. FITC-labeled DIg (6.25 μg) was mixed with 15 μg each of dextran of different molecular weight and the percentage increase in fluorescence from that of untreated FITC-labeled DIg was measured. Values are mean ± SD of four DIg samples for each dextran variety and 1-O-methyl-α-D-glucoside (25 mM) was used as control

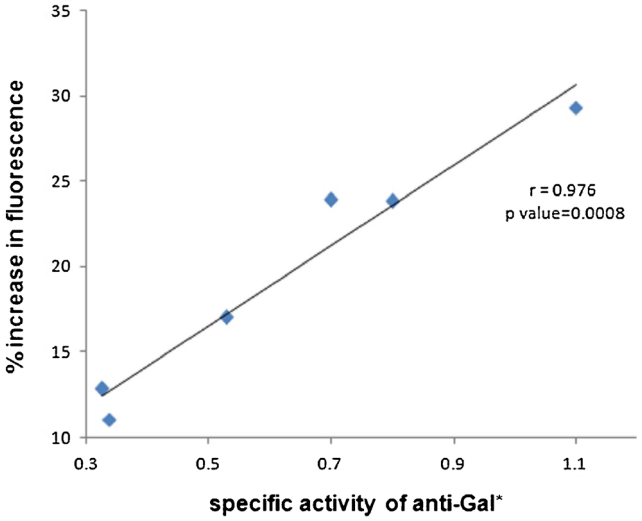

Fig. 3 Relation between fluorescence increase produced in FITC-labeled anti-Gal by thyroglobulin and specific activity of the antibody sample. FITC-anti-Gal (6.25 μg) samples differing in specific activity was mixed with thyroglobulin (15 ug) overnight and the percentage increase in fluorescence over untreated conjugate measured. * Ratio of thyroglobulin- binding activity to immunoglobulin content in ELISA (see text)

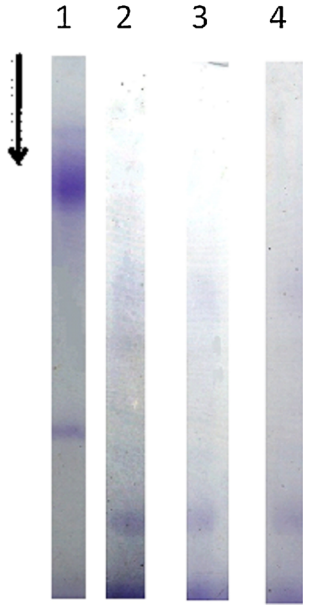

Fig. 4 7.5 % SDS-PAGE of papain-treated ABG without β-mercaptoethanol treatment. Papain concentrations (percentage of antibody protein) used to obtain samples run in lanes 1 to 4 were : 0; 2.5, 5 and 10 % respectively

enhancement upon antigen binding could be used as a tool to measure the antigen binding affinity of the antibody.

Fc Conformational Changes upon Antigen Binding is Responsible for Fluorescence Enhancement

To ascertain the contribution of conformational changes in Fc towards antigen induced fluorescence enhancement in FITC-labeled antibodies, effect of removal of Fc part of antibody by papain digestion was studied. Treatment with 2.5 % papain under conditions described completely digested the antibody since the antibody sample purified by affinity chromatography after enzyme digestion had no intact IgG as shown by poly-acrylamide gel electrophoresis in presence of SDS (Fig. 4). When ABG antibody subjected to papain digestion and later purified by affinity chromatography was captured on micro-plate coated antigen (TiC) and probed for presence of Fc re-gion using HRP-labeled anti-Fc antibody, the response was considerably lower than that of intact antibody (Fig. 5). The marginal response towards anti-Fc antibody observed even after digestion with 5 and 10 % papain concentrations could be attributed to less than absolute Fc specificity of the probing antibody used, rather than any remaining undigested antibody given the electrophoretic mobility in Fig. 4. Antibody devoid of Fc part produced, on an average, nearly 63 % less fluores-cence than its native counterpart following binding of specific ligand (TiC) (Fig. 6).

Plasma anti-carbohydrate antibodies were used for the present study for the following reasons: a) source of these antibodies (out-dated plasma from blood transfusion department) is readily available; b) plasma contains several anti-carbohydrate antibodies, each having distinct but defined specificity for respective carbohydrate antigens; c) mono- or divalent sugar antigens as well as macromolecular polysac-charide or glycoconjugate antigens are easily available for the three different plasma antibodies used; d) protection of antigen binding site by small mono- and disaccharide ligands enabled limiting of fluorescent labeling to Fc part; e) single step affinity chromatography protocol involving binding to natural or synthetic polysaccharide moieties and subsequent elution using dialyzable mono- or divalent sugars makes their

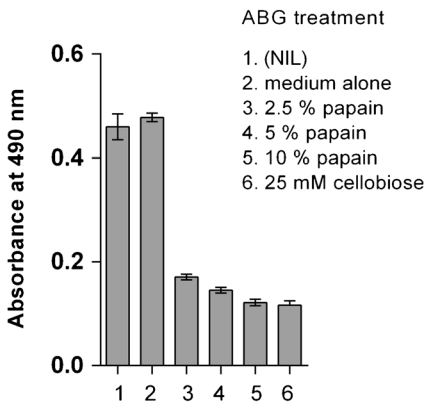

Fig. 5 Fc removal after papain digestion was checked by capturing the antibody (ABG) after enzyme digestion (2 ug) on microplate-coated TiC (2 ug per well) and bound antibody probed using HRP conjugate of Fc-specific anti-human IgG. Untreated ABG with or without inhibitory sugar (25 mM cellobiose) was also used. (Values are mean ± SD of 3 samples)

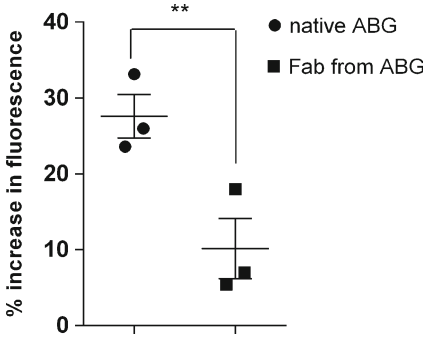

Fig. 6 Effect of papain digestion of ABG on fluorescence enhancement produced by ligands. Untreated ABG (6.25 ug; equalent to 3 μg Fab) or Fab (3 ug) from papain-treated ABG was mixed with 15 μg ligand and fluorescence enhancement was measured. Values are mean ± SD of 3 pairs. P value for the difference in fluorescence enhancement between intact antibody and Fab=0.0045

isolation in pure form much easier than that of other antibodies, and finally f) being naturally occurring antibodies apparently involved in anti-microbial and/ or anti-cancer defence of the body, characteristics of these antibodies elucidated here assumes clinical relevance. Fluorescence ligands attached covalently to amino acid residues at ligand binding sites have been used in the past to monitor ligand induced conformational changes in proteins. Aminophenoxazole maleimide coupled to cysteine group of ligand binding site has been used as fluorescent probe to detect conformational changes in β2 adrenergic receptor [25]. Recently fluorescence of antibody Fab fragment flurolabeled at its variable region has been shown to increase with antigen binding due to antigen- dependent release of fluorescence quenching [26].

Unlike the above examples the present protocol measures fluorescence of FITC tags in one region of a protein consequent to ligand binding at a distantly located site in the same molecule. FITC coupling of each of the three antibodies used here was done in presence of specific sugar in order to protect the antigen binding site from accepting FITC moieties so that maximum amount of FITC coupling takes place in the Fc part of antibody. Most of the conformational changes in the antibody consequent to antigen binding takes place away from the antigen binding site [3]. Oda et al. [7] have reported that antigen binding causes allosteric conformational changes in the constant domains as well. The signals resulting from antigen binding have been shown to cause considerable conformational changes in CH1-CH2 domains [7, 21] which are far away from the antigen binding site. It is reasonable to conclude that these conformational changes are responsible for the fluorescence enhancement in FITC-labeled antibodies upon antigen binding observed in the present results. Fluorescent tags attached to proteins are known to change their emitted fluorescence with changes in the microenvironment following conformational shifts [27]. Fluorescence enhancement upto about 10 % in Fab following antigen binding observed above could arise from FITC labeling of amino acid residues on the Fab region beyond the binding site of the protecting saccharide present during labeling, since conformational change in Fab region is also reported to accompany antigen binding [9].

Evidences for conformational change in Fc part consequent to occupation of binding site in Fab by large antigens include i) Fc of antigen-bound antibody getting receptive to complement and Fc receptors and ii) Fc:Fc association observed in immune complexes [28]. On any given antibody the extent of Fc activation and the ensuing complement fixation increased with the binding constant of the antigen used, indicating that energy of antigen binding was used to activate complement binding site of antibody [3]. Several lysine residues in IgG1 heavy chain such as at positions 322 and 326 in the Fc region and at positions 234 and 235 at the hinge region are crucial in recognition of the antibody by complement (C1q) following antigen-induced activation [29]. Since lysine residues are mainly used for covalent attachment of FITC it appears certain that conformational shift accompanying antigen binding would change the microenvironment around FITC moieties offering them more freedom to attain and dissipate energy resulting in greater fluorescence emission.

Conclusions

In the practical front the present results suggest that FITC-labeled antibodies may be used for detection as well as quantification of antigens in plasma or other biological fluids, therapeutic preparations or in culture supernatants of pathogens that secrete antigens. Further, data here also offer a way to compare the antigenic potential of candidate ligands present in food, pathogenic microbes or therapeutic preparations towards any given antibody. Naturally occurring antibodies induced apparently by gut bacteria are known to recognize complementary structures on macromolecules of dietary, bacterial or even host origin, forming immune complexes capable of causing inflammatory injury to host tissues [30, 31]. Two variables that dictate this pathobiology viz. specific activity of antibody and structural heterogeneity of antigens can be monitored by the present protocol. For instance our preliminary (unpublished) data reveal that the genetically determined size of human lipoprotein (a) determines its affinity for circulating anti-Gal antibody which is known to accommodate the serine- and threonine-rich peptide sequences of the lipoprotein as surrogate antigen [10]. To sum up, conformational shift in antibody Fc region consequent to binding of macromolecular antigens could be measured in terms of fluorescence enhancement of FITC label at Fc region. This enhancement increased with the affinity of antigen for a given antibody and with the specific activity of antibody for a given antigen.

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Conflict of Interest None

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