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Short communication

Horse IgG- and ostrich IgY-F(ab′)$_2$ groups have different affinities for mice erythrocytes and lymphocytes. Implications for avian immunoglobulin therapeutic usefulness

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ABSTRACT

We used high sensitivity and resolution fluorescence microscopy to study the interaction of ostrich IgY, horse F(ab′)$_2$ and horse IgG with mice lymphocyte and erythrocyte plasma membrane. The immunoglobulins were labeled with fluorescein isotiocyanate (FITC). Our results show an interaction of IgY with lymphocyte plasma membrane which does not result in endocytosis of the labeled protein. Less IgG and its F(ab′)$_2$ fraction bind to lymphocytes, and this binding seems to be followed by endocytosis producing a diffuse cytoplasmic fluorescence in most lymphocytes exposed to FITC-IgG or FITC-F(ab′)$_2$. Cytoplasmic fluorescence resembling FITC was not observed in lymphocytes exposed to FITC-IgY. Receptors in the erythrocyte membrane also differentiate between avian and horse Ig; while erythrocytes exposed to horse Igs became intensely fluorescent for at least 5 h, no erythrocyte labeling occurred when FITC-IgY was used. Our results suggest that IgY may be a stronger activator of adaptive immunity than horse IgG in mammals. Adaptive immunity against IgY is detrimental to its IV therapeutic use in humans and other mammals.

1. Introduction

The use of immunoglobulins has a growing significance in medicine. The production of antivenoms is of crucial importance to treat spider, snake and scorpion envenomings all over the world. The first antivenom for human use was prepared by Calmette (1894a, b) based on earlier studies by von Behring and Kitasato (1890) relating to the treatment of diphtheria and tetanus. Ever since Calmette's pioneering work, antivenoms are mostly produced in hyperimmunized mammals. A similar situation is true for monoclonal antibodies used as antineoplastics which are all of mammalian origin, human or mice. Murine monoclonal antibodies may be humanized, i.e., genetically engineered to produce more human-like antibodies (Chadd and Chamow, 2001; Rang et al., 2007).

Klemperer (1893) showed that yolk extracts from eggs laid by hens hyperimmunized against tetanus toxin were able to protect mice against this toxin. In favor of the use of IgY are the easiness and low cost of handling poultry, and that IgY reactivity with human FC receptors is low, which should result in a lesser chance of severe adverse reactions (Larsson et al., 1992, 1993; Calzado et al., 2003). Still, IgY are very heterologous proteins, phylogenetically very remote from mammals. Phylogenetic analysis of immunoglobulin γ

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heavy chain amino acid sequences from various mammalian species and equivalent immunoglobulin υ chains from amphibia and birds, shows that heavy chains of amphibia and birds are a separated cluster from mammalian γ chains. Analysis of Ig γ chains, suggest a close phylogenic relationship between mammalian immunoglobulins such as rat, mice, rabbit, human, chimpanzee, macaques, pig, sheep, cattle, camel and horse immunoglobulins (Wagner et al., 2002).

It has been previously shown that human immunoglobulins contains high levels of antichicken IgG, which could significantly reduce the efficiency of antivenoms (Sevcik et al., 2008). This problem would be very severe for antivenoms based on IgYs and could induce untoward reactions against antivenoms in patients previously sensitized against the host where the antivenom was produced (Sevcik et al., 2008). Here we use 3D deconvolution fluorescent microscopy (McNally et al., 1999) to follow horse FITC-IgG and FITC-F(ab′)$_2$ as well as ostrich (*Struthio camelus*) FITC-IgY injected i.v. to mice as a model to gain insight on the interaction of lymphocytes with avian and mammalian immunoglobulins and on their relevance in triggering adaptive immunity against them.

2. Material and methods

2.1. Sources of immunoproteins

F(ab′)$_2$ was commercial Venezuelan *Tityus* scorpion horse (*Equus caballus*) antivenom (Suero Antiescorpionico™, Biotecfar, Facultad de Farmacia, Universidad Central de Venezuela, Caracas). Separation of IgG directly from horse plasma was done with a modification of the method of Sheoran and Holmes (1996) using FPLC, in our case. Briefly, 100 mg of Ig obtained from horse plasma by caprylic acid precipitation were loaded on a XK 16/10 Q-Sepharose column (160 × 10 mm, Amersham Bioscience, Uppsala, Sweden), and subjected to a lineal gradient from the equilibrium buffer (25 mM Tris–HCl, pH 8.5) to 0.6 M NaCl in 70 min at a flow rate of 1 ml/min.

To separate ostrich IgY one egg yolk (≈150 ml) was carefully washed with cold water (4 °C) and mixed 50% v/v with cold water and well blended with a stirrer. Chloroform 1:1 v/v was then added in cold while stirring until a uniform creamy mixture was produced. The creamy suspension was centrifuged at 10,000 *g* at 4 °C for 40 min. The intermediate and chloroformic layers were discarded, and the aqueous layer, containing IgY, was aliquoted and stored at 4 °C with 0.1% sodium azide.

2.2. Labeling immunoglobulins

Labeling F(ab′)$_2$ with FITC was done as indicated by Sevcik et al. (2004) using 2 ampules (10 ml) F(ab′)$_2$, which were mixed with 4.5 ml of 50 mM sodium borate buffer, pH 9.6, and 0.5 ml of a solution of 4% FITC in acetone was added under vigorous agitation in a vortex stirrer. The tube was then completely covered with aluminum foil and the mixture was left at room temperature (≈22 °C) for 6 h and then at 4 °C overnight. The mixture was then washed by ultra filtration with 150 ml of 50 mM sodium borate buffer, pH 8, through an AMICON-Millipore membrane (PBDK type, 50 kDa cut-off, 25 mm diameter) under nitrogen pressure (4 bar) at 4 °C in darkness. The labeled material was thus reduced to between 2 and 3 ml and washing buffer was added to make 5 ml. The final product was aliquoted and stored at −80 °C Labeling of IgG and ostrich IgY followed the same protocol, with the following minor changes. Twenty four milligrams of IgG or 20 mg of IgY were diluted in sodium borate buffer (pH 9.6) and the remaining procedure was done as explained above.

2.3. Animal source and care

Male white mice (Balb/C mice, 25–30 g, $n = 8$, IVIC strain) were obtained from the central IVIC's animal facility and kept with food and water *ad libitum* in our Center's animal room. Mice were anesthetized by injecting SQ 100 μL of a mixture 1 ml of ketamine solution (100 mg/ml), 1 ml of xilazine solution (100 mg/ml) and 4.6 ml of sterile saline (NaCl 0.9%), which is equivalent to 50 mg/kg of each drug. Immediately, a catheter was introduced in a tail vein for administration of saline (controls), FITC-immunoproteins [≈2.9 μg/g, FITC-IgG, FITC-F(ab′)$_2$ or FITC-IgY]. Fifteen minutes, 1 or 5 h after injection the animals were anesthetized as indicated above, subject to cardiac perfusion with sterile saline, and organs were fixed by perfusion with Bouins's fluid. Lymphocytes were observed in blood vessels of kidney sections; samples were immersed in Bouins's fluid, for 24 h at room temperature (Cloutier et al., 2006).

2.4. Tissue sections preparation and staining

Tissues were washed with water, dehydrated with ethanol 70% v/v, clarified with xylene and embedded in Paraplast®. Five micrometer tissue sections dewaxed in xylene, and re-hydrated with PBS, were discolored with 0.5% w/v ammonium acetate to eliminated picric acid, and washed with PBS. To reduce auto fluorescence the slides were dehydrated for 1 h in 70% ethanol supplemented with 0.25% NH_4OH, then rehydrated with 50% ethanol for 10 min and transferred to PBS (Baschong et al., 2001; Viegas et al., 2007). Tissue sections were incubated in darkness for 10 min with the nuclear dye 4′,6′-diamino-2-phenylindole (DAPI; 1 μg/ml) in PBS and were washed with PBS during 1 min and counterstained with Evans Blue (EB, 0.01% in PBS) for 1 min (Cloutier et al., 2006), washed repeatedly with PBS, mounted with a coverslip using Immu-Mount® (Thermo Scientific, USA).

2.5. Fluorescence microscopy equipment

Sections were observed using Nomarski differential interference contrast (DIC) and 3D deconvolution epifluorescence on an automated upright microscope model DM6000 B (Leica Microsystems, Wetzlar, Germany) a Leica HCX PL APO 100×/1.4, oil (refractive index 1.515 at 23 °C) objective was used for fluorescence observation. Epifluorescence filter cubes used were (emission and excitation wavelength used between parentheses): Leica A4 (359/461 nm) for DAPI, Leica L5 (495/519 nm) for FITC and Leica N3 (595/613 nm) for EB. Images were captured with a high