

AVIAN IgY BINDS TO A MONOCYTE RECEPTOR WITH IgG-LIKE KINETICS DESPITE AN IgE-LIKE STRUCTURE.

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An ancestor of avian IgY was the evolutionary precursor of mammalian IgG and IgE, and present-day chicken IgY performs the function of human IgG despite having the domain structure of human IgE. The kinetics of IgY binding to its receptor on a chicken monocyte cell line, MQ-NCSU, were measured, the first time that the binding of a non-mammalian antibody to a non-mammalian cell has been investigated ($k_{+1} = 1.14 \pm 0.46 \times 10^5 \text{ mol}^{-1} \text{ sec}^{-1}$, $k_{-1} = 2.30 \pm 0.14 \times 10^{-3} \text{ sec}^{-1}$ and $K_a = 4.95 \times 10^7 \text{ M}^{-1}$). This is a lower affinity than that recorded for mammalian IgE/high-affinity receptor interactions ($K_a \approx 10^{10} \text{ M}^{-1}$), but is within the range of mammalian IgG/high-affinity receptor interactions (human: $K_a \approx 10^8\text{-}10^9 \text{ M}^{-1}$; mouse $K_a \approx 10^7\text{-}10^8 \text{ M}^{-1}$). IgE has an extra pair of immunoglobulin domains compared with IgG. Their presence reduces the dissociation rate of IgE from its receptor twenty-fold, thus contributing to IgE's high affinity. To assess the effect of the equivalent domains on the kinetics of IgY binding, IgY-Fc fragments with and without this domain were cloned and expressed in mammalian cells. In contrast to IgE, their presence in IgY has little effect on the association rate and no effect on dissociation. Whatever the function of this extra domain pair in avian IgY, it has persisted for at least 310 million years and has been co-opted in mammalian IgE to generate a uniquely slow dissociation rate and high affinity.

IgY is the principal serum antibody of amphibians, reptiles and birds and shares a common ancestor with both mammalian IgG and IgE (1,2). The cDNA sequence of the chicken ϵ heavy chain (3) reveals that, like more

basal amphibian IgY (4,5), avian IgY contains a domain pair (C ϵ 2) that has been conserved in mammalian IgE (as C ϵ 2) but truncated to form the "hinge" region in mammalian IgG (3). Thus, an orthologous domain pair must have existed in the common (IgY-like) ancestor prior to its duplication and subsequent divergence in the mammalian lineage. Avian IgY is the closest extant protein to this ancestor and therefore the most logical choice for study of the evolution of modern IgG and IgE from an ancient IgY-like ancestor.

IgG, the principal mammalian serum antibody, aggregates and facilitates opsonisation of antigens, activates complement, and provides protection for the foetus following transport across the placenta. IgE does not activate complement nor cross the placenta, but can sensitize effector cells (principally mast cells and basophils) and typically mediates anaphylactic reactions (6). It is well known for its involvement in allergic disease, but probably also provides defence against parasitic infections (7).

Avian IgY appears to combine the functions of mammalian IgG and IgE. As the major serum antibody it provides defence against infection (8), but also mediates anaphylaxis (9). Chicken mast cells activated by IgY are responsible for local anaphylactic reactions in the gut, which play a role in defence against protozoan infections (10,11). Antibody-dependent hypersensitivity and fatal systemic anaphylactic shock have also been demonstrated in chickens (9,12,13), and shown to be mediated by basophils (14), which are much more numerous than mast cells in birds (in contrast to mammals in which the reverse is true)(15). These phenomena constitute indirect evidence for the presence of IgY receptors on both chicken monocytes/macrophages and basophils.

The ν heavy chain of IgY consists of four constant immunoglobulin (Ig) domains and one variable Ig domain (3), as in mammalian IgE. It is clear from sequence comparisons that after the divergence of the synapsids 310 million years ago (mya) (16,17), but before the divergence of the non-eutherian mammals 166 mya (18), duplication of the ν -gene occurred; this was followed by differentiation into the two Ig classes unique to mammals, IgG and IgE, as indicated by their presence in monotremes (19,20). In IgG, the second domain of the heavy chain constant region condensed to form the hinge polypeptide (3), whereas in IgE the domain was retained as C ϵ 2 so that, like IgY, the Fc fragment consists of three constant Ig domain pairs. The two inter-heavy-chain disulphide bridges in the C ϵ 2 domain are present also in C ν 2 at the same locations, and in the hinge of IgG (Fig.1).

Human mast cells and basophils express the high affinity IgE receptor (Fc ϵ RI) which has an affinity for IgE that is uniquely high: $K_a \approx 10^{10} \text{ M}^{-1}$ (7). We have shown, through our studies with IgE, IgE-Fc and a sub-fragment of IgE-Fc consisting of only the C ϵ 3 and C ϵ 4 domains termed Fc ϵ 3-4 (21), that the high affinity is due to the slow dissociation rate from Fc ϵ RI (22,23), and that the C ϵ 2 domains are in part responsible (24). With a half-life on tissue mast cells of 2-3 weeks (25), IgE can effectively and persistently sensitize these cells for rapid degranulation upon encounter with antigen (allergen). The rapidity of degranulation is responsible for the life-threatening response of anaphylaxis, making an understanding of the mechanism by which the C ϵ 2 domains alter the dissociation rate of IgE from its receptor a medically important issue.

C ν 2-like domain pairs have persisted since their first appearance in a common ancestor that unites all terrestrial vertebrates. To assess their effect on the antibody-Fc receptor interaction, we have expressed two avian IgY-Fc fragments that differ only by the presence (Fc ν 2-4) or absence (Fc ν 3-4) of the C ν 2 domain pair, and measured their binding to the chicken monocyte cell line MQ-NCSU, as well as that of whole IgY. This is the first report of the cell-binding properties of a non-mammalian antibody, and also addresses the question of whether the "extra" C ν 2 domain pair

in IgY necessarily leads to a high-affinity interaction.

EXPERIMENTAL PROCEDURES

Cloning of Fc ν 2-4 and Fc ν 3-4—There is no intron between the CH1 and CH2 exons in chicken (26). Hence, the N-terminus of chicken C ν 2, Val 231 by the numbering system of Suzuki and Lee, (27), was identified by a Clustal W alignment of the sequences of the heavy chain constant region of human IgE (28) and of duck IgY (29), with that of chicken IgY taken from Parvari *et al.* (3). The second residue of C ϵ 2 is a cysteine that in IgE forms an intra-chain disulphide bond with C ϵ 1; it was therefore mutated to an alanine in Fc ϵ 2-4 and has similarly been mutated in Fc ν 2-4. The N-terminal 4 amino acids of IgY-Fc were Asp-Ile (from an EcoRV site) Val-Ala.

Fc ϵ 3-4 included the residues Asp-Ile from an EcoRV restriction site and two amino acids from C ϵ 2 one of which, a cysteine, was thought to be required to form a functionally important inter-chain disulphide bond (21). For this reason, a similar Fc ν 3-4 with an N-terminus including Cys 340 was cloned and expressed, but the protein formed intra-chain disulphide bonds with the "extra" Cys347 in chicken IgY (Fig.1). A Cys347Ser mutant led to expression of a functional protein, Fc ν 3-4, which has been used for the study reported here.

The polymerase chain reaction was used to engineer EcoRV and PmeI sites onto the 5' and 3' termini respectively of both Fc ν 2-4 and Fc ν 3-4 cDNA from a chicken splenocyte library (kindly donated by Dr. John Young, Institute of Animal Health, Compton U.K.). Using a holding vector, a PmeI site was introduced after the stop codon of IgE-Fc so that using EcoRV and PmeI, IgY-Fc could be exchanged for IgE-Fc, and then inserted into the pRY vector as a HindIII-HindIII cassette (30). Vectors were transfected into NS0 mouse myeloma cells for production of stable clones expressing Fc ν 2-4 or Fc ν 3-4.

Purification and characterization of IgYFc fragment—NS0 mouse myeloma cell supernatants were purified by elution from an anti-IgY-agarose affinity column (Gallus Immunotech, DAIGY-AGA) with 0.2M glycine pH 2.5, and dialyzed against phosphate buffered saline (PBS)

containing 0.05% sodium azide. Protein concentrations were measured in a Cary 50 spectrophotometer (Varian). The molar extinction coefficients for IgY, Fc γ 2-4 and Fc γ 3-4 were calculated using ProtParam software (31) and were 209125, 90965 and 62505 mol⁻¹cm⁻¹ respectively. Proteins were analyzed on denaturing SDS gels containing 12% acrylamide and the buffer system of Laemmli (32). Western blots used a semi-dry transfer method (33) and anti-IgY Horse Radish Peroxidase (Promega, G135A) was used for detection.

Cell lines—The MQ-NCSU cell line was provided by Dr. M.A. Qureshi, National Program Leader, Animal Genetics, USDA Cooperative State Research Education and Extension Service, Washington DC 20024 (34). Cells were grown at 37°C in modified LM-Hahn medium omitting foetal bovine serum and chick serum, and thereby exogenous IgY, but including 8% ultra-low IgG foetal bovine serum (Invitrogen, 16250-078).

Flow cytometry (FACS)—FACS was used to detect immunostained cells following their incubation with specific antibodies and/or antibody conjugates. In all experiments, cells were washed (by centrifugation at 1000xg for 5 minutes) and incubated for 10 minutes in PBS containing 1% bovine serum albumin (BSA) (Sigma, A7030) before staining for 1 hour at 4°C with chicken IgY-(Fab)₂ (Rockland, 003-0104), (Fab)₂ + whole IgY, IgY or buffer before incubation with a mouse monoclonal antibody (Sigma, C-7295), which was found to be an anti-C γ 2*, and detection by anti-mouse fluorophore-conjugated secondary antibodies (Dako, F0479). Cells were washed twice with PBS-BSA both before and after all antibody incubation steps, and fixed in PBS + 4% formaldehyde (Sigma, F1635) before analysis in a FACS Calibur instrument (BD Biosciences).

Radioiodination of antibodies and antibody fragments—Affinity purified IgY (Stratech, 003-000-003) and IgY-Fc were radiolabelled with ¹²⁵I (PerkinElmer, NEZ033A001MC) using the chloramine-T method (35). Desalting spin columns (Pierce, 89849) were used to remove unincorporated ¹²⁵I and exchange the radiolabelled IgY or IgY-Fc into PBS/0.05% sodium azide, following the manufacturer's protocol. The specific activity in cpm/ μ g of each radioligand was

determined by estimation of the protein concentration (by UV spectrophotometry) and cpm of radioactivity, using a Wizard 1480 gamma counter (PerkinElmer).

Cell binding assays—MQ-NCSU cells were washed in PBS/1% BSA/0.05% sodium azide, pH 7.4 and incubated at 23°C for ten minutes before resuspension at 10⁷ cells/ml. In experiments to determine the association rate constant, duplicate samples of 10⁶ cells were incubated with 10nM ¹²⁵I-labelled IgY, Fc γ 2-4 or Fc γ 3-4 at 23°C. At regular intervals, cells were centrifuged through 200 μ l of phthalate oil (36) and the radioactivity of the cell pellet determined using a Wizard 1480 gamma counter (PerkinElmer). The initial rate of association was determined from a linear fit of cpm versus time. Standard errors were generated by the fitting procedure but, due to uncertainty in number of receptors per cell (see Discussion), larger errors have been added to the final values.

Dissociation experiments were performed using the method of Kulczycki and Metzger (37), but with all steps carried out at room temperature; cells were first incubated with 10nM ¹²⁵I-labelled IgY, Fc γ 2-4 or Fc γ 3-4 for 1 hour, then resuspended in an excess of unlabelled IgY and their radioactivity assayed as above. Non-specific binding was measured by incubating cells with an excess (350nM) of unlabelled IgY for 1 hour at room temperature before addition of 10nM labelled IgY and the gamma counts obtained subtracted from the total bound counts. The mean number of receptor sites per MQ-NCSU cell was determined by titrating to saturation with ¹²⁵I-labelled IgY or Fc γ 2-4 of known specific activity. Dissociation rate constants were determined by both non-linear regression analysis from a plot of cpm versus time (according to equation 20, p88, in Goodrich and Kugel)(38) and also from linear regression analysis of a plot of ln (cpm) versus time.

RESULTS

The much higher affinity of IgE for its receptor on mast cells compared to IgG for any of its receptors, and the role of the "extra" C ϵ 2 domain pair in IgE, prompted this study of chicken IgY, the closest extant immunoglobulin to the ancestor that evolved into all three antibodies.

In a preliminary FACS experiment it was established that chicken serum IgY binds to MQ-NCSU cells in an Fc-dependent manner: IgY Fabs at 400x molar excess over whole IgY were unable to bind to cells or to inhibit IgY binding (Fig.2). The binding constants determined in the studies described below are thus for IgY and IgY-Fc binding to an IgY-Fc receptor on a chicken monocyte cell line.

The action of papain on IgY does not give rise to an Fc γ 2-4 fragment since the C γ 2 domain is digested away (27). Hence, in order to assess the role of C γ 2, Fc γ 2-4 and Fc γ 3-4 were cloned and expressed as recombinant proteins. Purified IgY (Fig.3, lane 1), recombinant Fc γ 2-4 (lane 2), and Fc γ 3-4 (lane 3), both unreduced (Fig.3A,C) and reduced (Fig.3B,D) were electrophoresed and stained with Coomassie Brilliant Blue (Fig.3A,B) or transferred to nitrocellulose and stained with anti-IgY HRP conjugate (Fig.3C,D). Reduced heavy chains gave rise to a single band (Fig.3C, lanes 1,2,3) suggesting that the small amount of heterogeneous-sized material in unreduced samples was due to the formation of non-native disulfide bonds on exposure to 100°C in SDS buffer (39) and not to degradation or heterogeneity of glycosylation. Western blots of the same samples (Fig.3C,D) showed that the polypeptides are indeed IgY of approximately the expected molecular mass with only a minor band of Fc γ 3-4 monomer (Fig.3C, lane 3). All were glycosylated as shown by periodic acid Schiff staining* and all had essentially no free sulphhydryls per mole of IgY or its fragments, as determined by reduction and carboxymethylation*(40).

The measured rate constant for the dissociation of ^{125} I-IgY from MQ-NCSU cells was $2.30 \pm 0.14 \times 10^{-3} \text{ sec}^{-1}$ (Fig.4A). A plot of $\ln(\text{cpm})$ against time yields a straight line, showing that a single, zero order dissociation event is involved (returning a confirmatory value of $2.05 \pm 0.04 \times 10^{-3} \text{ sec}^{-1}$ for k_1 ; Fig.4B). The same value was obtained when biotinylated IgY, stained with streptavidin-R-phycoerythrin, dissociated from the cells after addition of unlabelled IgY*, suggesting that the derivatization of lysine with biotin, or tyrosine with iodine, does not perturb the IgY-receptor interaction.

In order to measure the rate of association of ^{125} I-IgY with MQ-NCSU cells, an estimate of the

number of receptors per cell was made. Measurements on two different days led to an estimate of 20,000, and the likely error in this value is discussed below. The value obtained for k_{+1} is $1.14 \pm 0.04 \times 10^5 \text{ mol}^{-1} \text{ sec}^{-1}$ (Fig.4C). The two rate constants lead to a value for $K_a = 4.95 \times 10^7 \text{ M}^{-1}$.

When the binding kinetics of the IgY-Fc fragments were determined, Fc γ 2-4 was found to display the same rate of dissociation as Fc γ 3-4 (Fig.5A), showing that the C γ 2 domain pair does not have a detectable effect on the rate of dissociation. The association rate constant for Fc γ 2-4 was $3.66 \pm 0.16 \times 10^5 \text{ mol}^{-1} \text{ sec}^{-1}$ and for Fc γ 3-4, $1.64 \pm 0.01 \times 10^5 \text{ mol}^{-1} \text{ sec}^{-1}$, which differ only by a factor of about two (Fig.5B). This is not a significant difference given the errors inherent in this technique (discussed below).

DISCUSSION

These studies determine for the first time the binding kinetics of IgY and its Fc fragments to cells, and demonstrate that the affinity is more similar to that of IgG for its high-affinity receptor, than IgE for its high-affinity receptor. Furthermore, the kinetics of the interaction are not affected by the presence or absence of the C γ 2 domain pair, in contrast to the effect that the C ϵ 2 pair has on the kinetics of IgE binding to Fc ϵ RI (24).

IgY dissociates from its receptor on the MQ-NCSU chicken monocyte cell line with a rate constant of $k_1 = 2.30 \pm 0.14 \times 10^{-3} \text{ sec}^{-1}$ at 23°C (Fig.4A). Although ten times faster than the dissociation of human IgG1 from the monocyte cell line U937 ($k_1 = 1.1 \pm 0.1 \times 10^{-4} \text{ sec}^{-1}$)(41), k_1 for IgY is close to the value $3.5 \times 10^{-3} \text{ sec}^{-1}$ (no error reported) measured for the dissociation of mouse IgG subclass 2a from the mouse macrophage cell line P388D (42). Dissociation of human IgE from (cord blood) mast cells is so slow by comparison, that even at 37°C, values of $6.05 \times 10^{-5} \text{ sec}^{-1}$ (43)(no error given) and $6.93 \pm 0.54 \times 10^{-5} \text{ sec}^{-1}$ (44) have been recorded.

A value of $1.14 \pm 0.04 \times 10^5 \text{ mol}^{-1} \text{ sec}^{-1}$ has been determined for the association rate constant of IgY with MQ-NCSU cells, although a much larger error is inherent in this value (and consequently the K_a) than in that of the

dissociation rate constant. The number of receptors per cell is a factor in the calculation, and it has been reported that Fc receptor number on human monocytes may vary on average by $\pm 25\%$ and up to 40% (45); the error in k_{+1} for IgY may therefore be as much as $\pm 40\%$. The value of k_{+1} for IgE binding to the Fc ϵ RI alpha chain expressed on CHO cells at 25°C is $3.1 \pm 0.3 \times 10^5 \text{ mol}^{-1} \text{ sec}^{-1}$ (30), and for human IgG1 to U937 cells is $2.1 \pm 0.2 \times 10^5 \text{ mol}^{-1} \text{ sec}^{-1}$ (41). Thus the association rate constant for human IgE differs from the value determined here for IgY binding to MQ-NCSU cells by a factor of only 2.7, in contrast to the difference in affinity, which is about 700-fold ($K_a = 4.95 \times 10^7 \text{ M}^{-1}$ for IgY; $K_a = 3.4 \times 10^{10} \text{ M}^{-1}$ for IgE) (30). The difference between IgY and IgE in their affinity for cells is therefore due almost entirely to a difference in their rates of dissociation (as it is for IgG compared with IgE). The affinity of IgY reported here is within the range of mammalian IgG/high affinity receptor interactions: e.g. human $K_a \approx 10^8\text{-}10^9 \text{ M}^{-1}$; mouse $K_a \approx 10^7\text{-}10^8 \text{ M}^{-1}$ (46).

The presence of the C ϵ 2 domain pair slows the rate of dissociation of IgE from Fc ϵ RI by a factor of approximately twenty, as shown by a comparison of the binding kinetics of two IgE fragments, one with this domain (Fc ϵ 2-4) and one without (Fc ϵ 3-4) (22,24). The influence of the C ν 2 domains on the kinetics of IgY binding to cells was therefore investigated using two corresponding IgY fragments, Fc ν 2-4 and Fc ν 3-4, differing only by the C ν 2 domain pair. The dissociation curves of Fc ν 2-4 and Fc ν 3-4 can be superimposed (Fig.5A), showing that unlike IgE-Fc (24), the presence of the C ν 2 domain pair has no effect on the rate of dissociation. Although the values for the association rate constants differ by a factor of 2.2 ($k_{+1} = 3.66 \pm 0.16 \times 10^5 \text{ mol}^{-1} \text{ sec}^{-1}$ for Fc ν 2-4; $k_{+1} = 1.64 \pm 0.01 \times 10^5 \text{ mol}^{-1} \text{ sec}^{-1}$ for Fc ν 3-4), the potential error in these values due to possible variability in cell receptor number of $\pm 40\%$, means that these values are not significantly different.

Although the C ν 2 domains of chicken IgY do not appear to contribute to receptor binding on monocytes, this does not preclude a role in, for instance, binding to mast cells or basophils. However, cutaneous anaphylaxis experiments in

chickens (9) have shown that IgY is not retained in tissue for as long as mammalian IgE (i.e. only for days rather than weeks), which suggests that the dissociation rate of IgY from mast cells is also more akin to that of IgG than IgE. A function for C ν 2 is likely however, as it has persisted in both mammals and birds for the 310 million years (my) since Synapsida and Reptilia diverged, and it has recently been estimated that DNA coding for a non-functional protein domain has a half-life of only 4my in the genome (47,48).

The association constant determined in the experiments described in this paper ($K_a = 4.95 \times 10^7 \text{ M}^{-1}$) is close to that determined for the interaction between chicken serum IgY and an extracellular domain of a member of the chicken leukocyte receptor complex (LRC), CHIR-AB1, as measured by surface plasmon resonance: $K_a = 3.70 \times 10^7 \text{ M}^{-1}$ (49). Since CHIR-AB1 mRNA was detectable in chicken M11 primary macrophages (49), it is likely that CHIR-AB1 is the molecule responsible for IgY binding to MQ-NCSU monocytes in the data presented here. One caveat in comparing these two values is that association constants obtained by cell binding and SPR sometimes differ: K_a for IgE binding to the Fc ϵ RI α chain displayed on CHO cells is 10x higher than the value obtained by surface plasmon resonance (22,24,30). Furthermore, additional polypeptide chains in the native, cell-bound receptor may influence affinity.

Another issue to consider is whether the MQ-NCSU cells express more than one receptor. The human monocyte cell line, U937, expresses a number of both IgG and IgE receptors, and MQ-NCSU cells may express receptors homologous to these, to which IgY may in principle bind. However, in studies of IgG binding to U937 cells, only one receptor, Fc γ RI, is detected, despite the presence of others, Fc γ RII and Fc γ RIII, because the dissociation rates are too fast to be measured (50). Similarly IgE binding to U937 cells detects neither CD23 unless induced (because it is expressed at such a low level) (51), nor Fc ϵ RI (which requires neuraminidase treatment to unmask it) (52). The kinetic data reported here for IgY binding to MQ-NCSU cells suggests that a single receptor is being detected.

Comparison of the association rate constants measured here in cell binding for IgY with those

determined by surface plasmon resonance (49) also shows good agreement: $1.14 \times 10^5 \text{ mol}^{-1} \text{ sec}^{-1}$ and $2.4 \times 10^5 \text{ mol}^{-1} \text{ sec}^{-1}$ respectively. These authors also measured the binding kinetics for an IgY-Fc fragment produced by papain digestion, consisting of a dimer of the C ν 3 and C ν 4 domains (49). Their value of $k_{+1} = 3.9 \times 10^6 \text{ mol}^{-1} \text{ sec}^{-1}$ for this fragment is 24x faster than that reported here, $k_{+1} = 1.64 \pm 0.01 \times 10^5 \text{ mol}^{-1} \text{ sec}^{-1}$, for the recombinant Fc ν 3-4. However, we also generated an Fc fragment by papain digestion and found, by N-terminal sequencing*(27), that a substantial proportion of the material consists of an incomplete C ν 3 domain; this may explain the discrepancy between the two k_{+1} values.

The studies of Viertlboeck *et al.* (49) and, we believe, the data reported here, concern the binding of an antibody to a receptor coded within the chicken LRC (on microchromosome 31). The mammalian LRC is a 1Mb gene cluster on human chromosome 19, consisting of 45 genes of which 30 encode Ig-like receptors that have mostly HLA class I molecules as ligands (53). In mammals, antibody Fc receptors are encoded mainly within the Fc receptor gene family (on human chromosome 1), but there are two examples of Fc receptors encoded within the mammalian LRC: the IgA receptor Fc α RI (CD89), and bovine Fc γ RIIb (54). There is a significant difference between the way in which human Fc α RI interacts with IgA, and how the 'classical' Fc receptors belonging to the FcR gene cluster interact with IgG and IgE. Crystal structures of these Fc-receptor complexes show that in both IgG and IgE, the binding site lies in a similar region at the N-terminus of the homologous C γ 2 and C ϵ 3 domains respectively, whereas IgA interacts with its receptor through a site close to the junction between C α 2 and C α 3 (55). (It is not yet known to which domains of IgG bovine Fc γ RIIb binds, but the interaction does involve domain 1 of the receptor, which is characteristic of LRC receptor interactions, in contrast to FcR, which involve domain 2). The chicken LRC sequence is incomplete, but already 103 genes highly related to each other have been identified (56); currently only CHIR-AB1 has a known ligand. As CHIR-AB1 is more closely

related to Fc α RI than the Fc receptors for IgG or IgE, chicken IgY may bind to its receptor in a similar manner to that in which human IgA binds to Fc α RI. If so, and the receptor binding site in IgY lies between C ν 3 and C ν 4 (and not at the N-terminal region of C ν 3), this would be consistent with our observation that the presence of C ν 2 plays no role in the binding kinetics.

Intriguingly, the FcR family in chicken appears to be represented by just a single gene (57,58), and our preliminary experiments show that this receptor does not bind IgY*. The mammalian FcR complex contains many receptor homologues whose ligands are not antibodies, and these receptors are thought to play a role in immunoregulation (59). These observations, and the data presented in this paper, lead us to speculate that Fc receptor function migrated from the LRC to the FcR locus (which subsequently expanded) during the last 310 million years. The migration of Fc receptor function may have been a consequence of the need to shift the binding site in the antibody Fc from the C ν 3/C ν 4 interface in the IgY-like ancestor of IgG and IgE, due to competition from bacterial Fc-binding proteins. Such proteins are known to bind to the C α 2/C α 3 interface in IgA (60) and the C γ 2/C γ 3 interface in IgG (61,62), thereby disabling the immune response by blocking FcR interactions. This selection pressure has led to co-evolution of IgA and its receptor (63); a receptor mutating too slowly to keep up with changing bacterial peptide sequences becomes redundant and may persist only as a pseudogene (as in rabbits)(63), or be eliminated altogether (as in mouse)(64). The IgY-like ancestor of IgG and IgE may have overcome this selection pressure by shifting the binding site to the N-terminal region of the domain that evolved into C γ 2 and C ϵ 3. Whatever the reason for the persistence of the C ν 2 domains in IgY and its ancestors, its presence in the ancestor of IgE served as a pre-adaptation for the evolution of an antibody domain (C ϵ 2) that contributes to the uniquely slow dissociation rate and high affinity for receptor, and the consequent advantages this confers for defence against parasites.

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FOOTNOTES

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The abbreviations used are: K_a , affinity constant, k_{+1} , association rate constant; k_{-1} , dissociation rate constant. IgY,E,G,A, immunoglobulin Y,E,G,A; IgY,E-Fc, Fc fragment of IgY,E; Fab, antigen-binding fragment; Fc γ 2-4, IgY-Fc fragment containing heavy chain constant domains 2,3 and 4; Fc ϵ 2-4, same for IgE; Fc γ 3-4, IgY-Fc fragment containing heavy chain constant domains 3 and 4; Fc ϵ 3-4, same for IgE; CH, constant heavy chain domain; C γ 2, C γ 3 second and third constant domains of IgY; C ϵ 2, C ϵ 3, same for IgE; Fc ϵ RI α , alpha chain of the high-affinity receptor for IgE; CD23, low affinity receptor for IgE; Fc γ RI, high-affinity receptor for IgG; Fc α RI or CD89, high-affinity receptor for IgA; CHIR-AB1, IgY receptor identified by Viertlboeck et al. (2007); LRC, leukocyte receptor complex; Mb, megabase; my, million years; mya, million years ago; FACS, flow cytometry; PBS, phosphate-buffered saline; cpm, counts per minute.

FIGURE LEGENDS

Fig.1

The domain structure of avian IgY and its mammalian homologues, IgG and IgE.

Schematic representations of the antibodies composed of variable (V) or constant (C) immunoglobulin domains that make up the heavy (H or $\gamma/\epsilon/\mu$) and light (L) chains are shown with Fab and Fc fragments circled. Inter-chain disulphide bonds are shown as solid (known) or dashed (putative) lines. The domain structure of IgG shown here is that of human IgG1, with the hinge region represented by a curved line linking C γ 1 and C γ 2. Other subclasses of IgG vary in hinge length and number of inter-heavy chain disulphides.

Fig.2

IgY binding to chicken monocytes is dependent on the Fc region

Prior to detection with mouse anti-C γ 2 (followed by anti-mouse Igs-FITC), cells (shaded histograms) were incubated in PBS-BSA containing either 20 μ M IgY-Fab alone (A), 50nM IgY alone (B) or 50nM IgY+20 μ M IgY-Fab (C). Control cells (open histograms) were incubated with PBS-BSA alone before detection. Values shown are ratios of mean fluorescence intensity of each sample to that of control cells.

Fig.3

Characterization of IgY and IgY-Fc fragments

Whole IgY from chicken serum and recombinant IgY-Fc fragments secreted from mouse myeloma (NS0) cells were purified by affinity chromatography and analysed using denaturing (SDS) 12% polyacrylamide gels, either stained with Coomassie Brilliant Blue (A & B) or Western blotted with polyclonal anti-IgY antibodies (C & D). Samples were either non-reduced (A & C) or reduced with 2% β -mercaptoethanol (B & D). **Lane 1**, IgY. **Lane 2**, Fc γ 2-4. **Lane 3**, Fc γ 3-4 [C340S]. Molecular weight markers are shown in kDa.

Fig.4

Binding of IgY to chicken monocytes.

Dissociation experiments were performed by incubating MQ-NCSU cells with 10nM ^{125} I-IgY from chicken serum for 1 hour followed by the addition of an excess of unlabelled IgY. Cells were spun through phthalate oil and their radioactivity (cpm) measured at regular intervals and normalised to the initial value. The dissociation rate constant was determined by both non-linear (A) and linear (B) regression analyses. Data from a single dissociation experiment are shown. Association experiments were

performed by measuring the radioactivity of cell pellets at regular intervals starting from the addition of 10nM ^{125}I -IgY. Mean data from two duplicate experiments are shown $\pm$ standard error of the mean. (C). All experiments were performed at room temperature.

Fig.5

Binding of IgY-Fc fragments to chicken monocytes.

Dissociation experiments were performed by incubating MQ-NCSU cells with 10nM ^{125}I -labelled IgY-Fc γ 2-4 ($\square$) or IgY-Fc γ 3-4 ($\blacksquare$) for 1 hour followed by the addition of an excess of unlabelled whole IgY. Cells were spun through phthalate oil and their radioactivity (cpm) measured at regular intervals and normalised to the initial value. Mean data from two duplicate experiments are shown $\pm$ standard error of the mean. (A). Association experiments were performed by measuring the radioactivity of cell pellets at regular intervals starting from the addition of 10nM ^{125}I -labelled Fc γ 2-4 ($\square$) or Fc γ 3-4 ($\blacksquare$). Mean data from two duplicate experiments are shown $\pm$ standard error of the mean. (B). Some error bars are too close to experimental points to be clearly visible. All experiments were performed at room temperature.

Figure 1

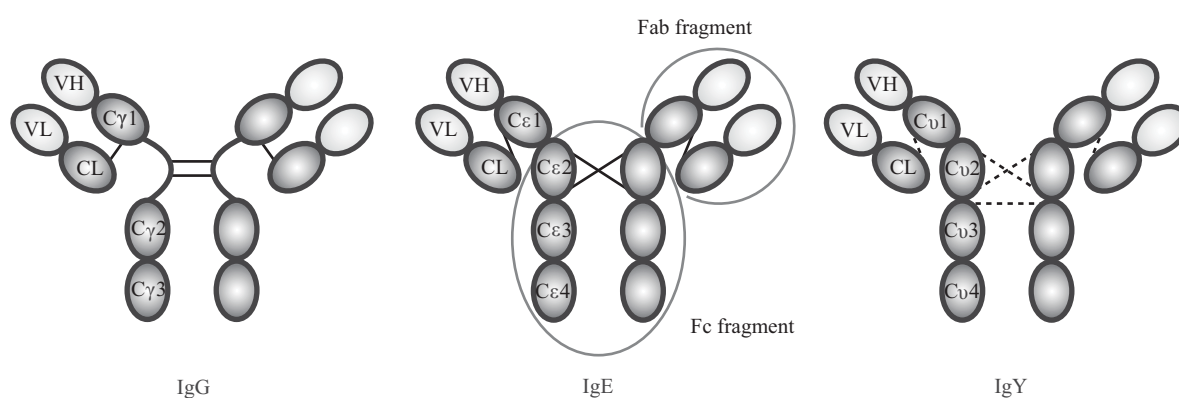


Figure 2

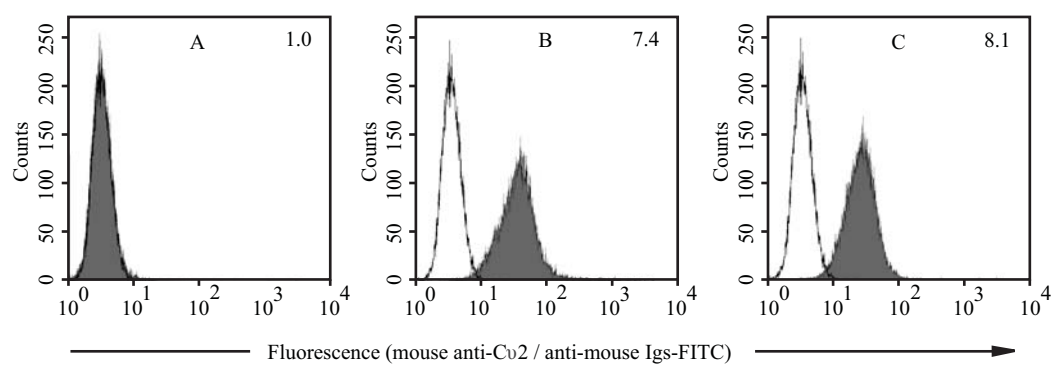


Figure 3

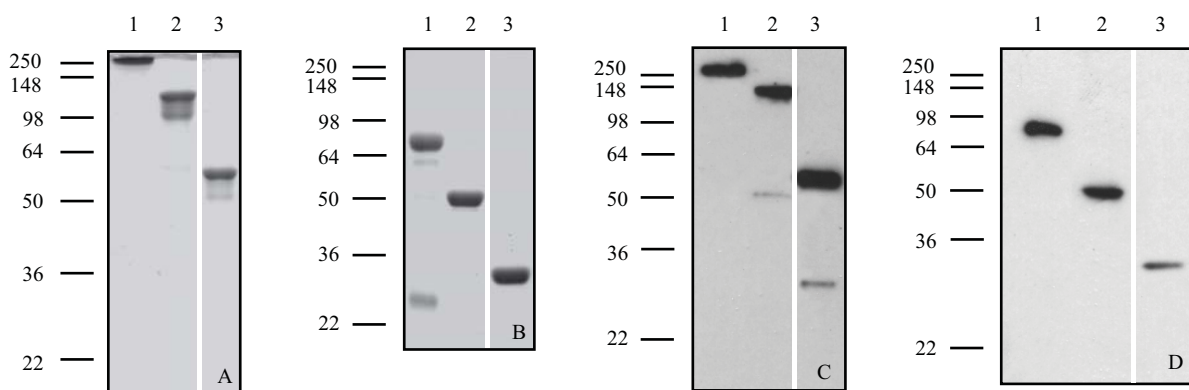


Figure 4

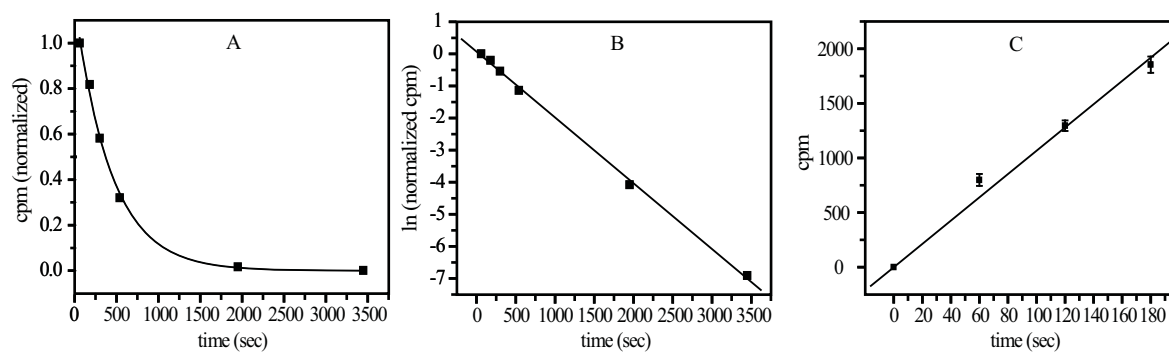
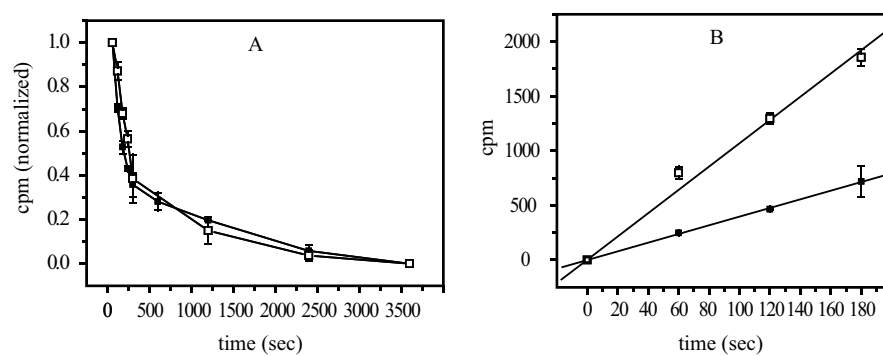


Figure 5



VOLUME 283 (2008) PAGES 19769–19780

An unusual TOM20/TOM22 bypass mechanism for the mitochondrial targeting of cytochrome P450 proteins containing N-terminal chimeric signals.

Hindupur K. Anandatheerthavarada, Naresh Babu V. Sepuri, Gopa Biswas, and Narayan G. Avadhani

This article has been withdrawn at the request of the authors. Further documentation regarding the figures is needed.

VOLUME 283 (2008) PAGES 16384–16390

Avian IgY binds to a monocyte receptor with IgG-like kinetics despite an IgE-like structure.

Alexander I. Taylor, Hannah J. Gould, Brian J. Sutton, and Rosaleen A. Calvert

On Page 16385, right column, the second line should read as follows: A C340S mutant led to expression of a functional protein, Fc γ 3–4, which has been used for the study reported here.

VOLUME 283 (2008) PAGES 14479–14489

Identification of extracellular signal-regulated kinase 1/2 and p38 MAPK as regulators of human sperm motility and acrosome reaction and as predictors of poor spermatozoan quality.

Tal Almog, Shlomi Lazar, Nachum Reiss, Nir Etkovitz, Eyal Milch, Nir Rahamim, Masha Dobkin-Bekman, Ronit Rotem, Moshe Kalina, Jacob Ramon, Arie Raziell, Haim Breitbart, Rony Seger, and Zvi Naor

Dr. Breitbart's name was misspelled. The correct spelling is shown above.

VOLUME 283 (2008) PAGES 8477–8485

Significance of interactions of low molecular weight crystallin fragments in lens aging and cataract formation.

Puttur Santhoshkumar, Padmanabha Udupa, Raju Murugesan, and K. Krishna Sharma

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Structural organization of photosynthetic apparatus in agranal chloroplasts of maize.

Elzbieta Romanowska, Joanna Kargul, Marta Powikrowska, Giovanni Finazzi, Jon Nield, Anna Drozak, and Berenika Pokorska

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