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Comparison between 'IgY technology' from chickens and 'IgG technology' from mice for production of tailor-made antibodies

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Abstract—Antibodies collected from chickens immunized towards a β-ketoalcohol, key intermediate in the synthesis of chrysanthemic acid, which have been shown to possess similar behavior to those elaborated from mice, offer the enormous advantages of no bleeding and access to large quantities of antibodies by easy extraction of the yolk of the eggs.

We recently described[1] that antibodies such as **ABM-OVA-1** and **ABM-KLH-2**, harvested from mice immunized towards the conjugates: **OVA-1** and **KLH-2** and selected over the related **BSA-1** and **BSA-2**, respectively (Fig. 1), do not recognize the bicyclic β-hydroxyketone (1*R*)-**4**, a key intermediate in one of our syntheses of chrysanthemic acid,[2] for which they have been elaborated (Scheme 1).

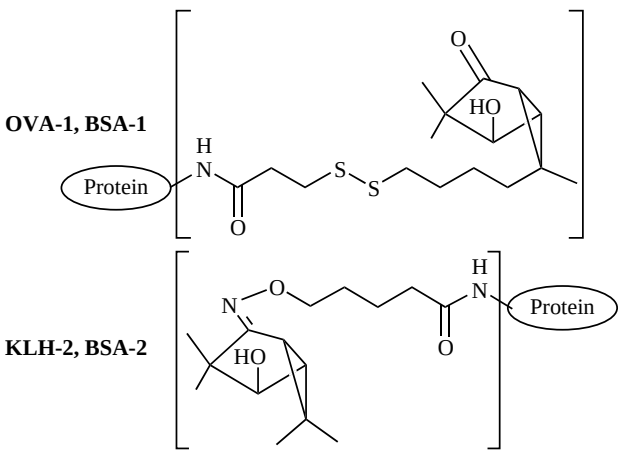

Figure 1. Conjugates used to harvest antibodies used in this work.

We also found that compounds **5** and **6c** (Fig. 2), which possess (i) the complete carbon framework present on the hapten; (ii) a side arm of sufficient length and (iii) the correct stereochemistry were recognized reasonably well (IC_{50}:>250 μM for **5** towards **OVA-1a** and IC_{50}: 8 μM for **6c** towards **ABM-KLH-2**).[1]

Further work performed on **ABM-KLH-2** clearly showed that several compounds somewhat related to (1*R*)-**4** were not recognized (IC_{50}>500 μM).[1] This is effectively the case of compounds missing (i) the bicyclic carbon framework; (ii) the alkyl chain on the oxime functionality such as **6a**; (iii) either the *exo*-(**7a**) or the *endo*-(**7b**) methyl group or whose stereochemistry differs (such as in **6c**′). Reasonable recognition (IC_{50}: 8 to 32 μM)[1] was however achieved with the families of compounds with side arms of slightly lower or higher length (**6b** and **6d**) or bearing different functional groups (**6e**).

In order to understand the reasons of such results and, as part of our strategy to achieve the recognition of **4** by tailor made antibodies, we decided to use chicken antibodies, especially IgY (yolk immunoglobulin), which are transferred by hens to their eggs.[3] These are the functional homologues of mammalian IgG whose structure nevertheless differs considerably from mammalian IgG.[4] For example chicken IgY heavy chain (~70 kDA) is heavier than mammalian IgG (~50 kDA) and therefore IgY technology is expected to behave differently from the IgG one previously used.[1]

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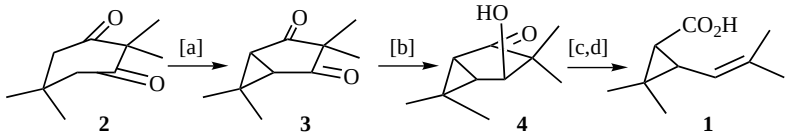
There are advantages to use IgY technology over the conventional one which uses mammals: (i) chicken housing is inexpensive; (ii) yolk/hen weight ratio is particularly good; (iii) eggs can be stored at 4°C for at least 1 year; (iv) large quantities of highly active IgY can be collected and produced[5,†] on a daily basis for more than 2 years from the same animal at condition of regular interval immunizations and last but not least; (v) IgY are easily separated from IgM and IgA, produced in the egg white, due to the natural compartmentalization of eggs.[6]

Moreover chicken eggs are relatively low cost 'green source' of polyclonal antibodies. Their use avoids the bleeding step, which causes remarkable distress to the animal involved and to a lower extent to the chemist and therefore it better conforms today's standards of animal-care.

Antibodies have been raised towards **KLH-2**[1] (Fig. 1) using keyhole limplet hemocyanin (**KLH**) as the carrier protein.[7] Typically one hybrid line hen aged 24 weeks was kept in individual wired cage with food and water provided ab libitum. All immunisations were administered in phosphate buffered saline solution (PBS) of QuilA adjuvant. The hen received 100 μg **KLH-2** (dissolved in 1 ml of 1/1 PBS/QuilA) intramuscularly into two sites at a two weeks interval (five times). Eggs (two)



Scheme 1. Synthesis of *cis*-chrysanthemic acid. (a) Br_2, *t*-BuOK, pentane–ether, 20°C, 1 h, 66%; (b) 1 mol equiv. $NaBH_4$, 1.1 equiv. $CeCl_3$, MeOH, 4 h, −78°C, 88%; (c) 1.2 equiv. TsCl, 2.5 equiv. Pyr., 0.2 equiv. DMAP, C H_2Cl_2, 16 h, 20°C, 95%; (d) (i) 6 equiv. KOH, aq. DMSO, 4 h, 70°C; (ii) H_3O^+, 69%.

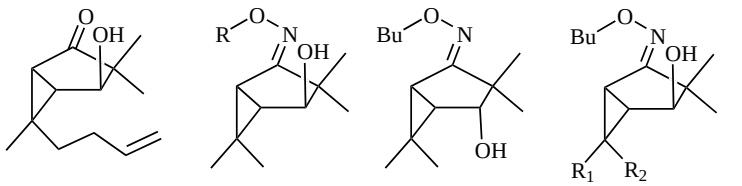


Figure 2. Set of compounds tested to define the recognition patern of antibodies.

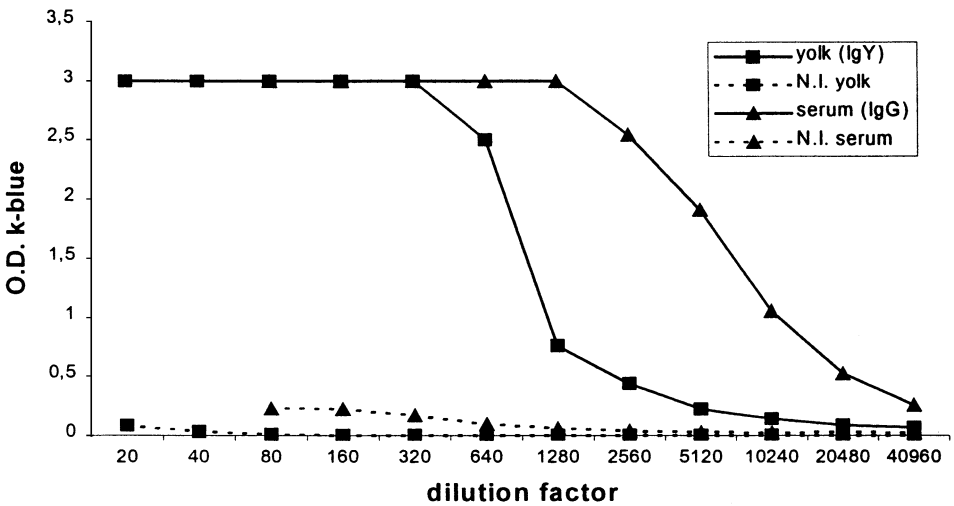


Figure 3. Indirect ELISA involving IgY and IgG collected from **KLH-2** (N.I. = non immunised).

† Up to 15–25 mg of IgY can be produced per ml of yolk in the case of hyper immunized hen and the amount of eggs of one hen in a month is equivalent to that in 500 ml of antiserum.

were collected from days 56 and 60 and stored at 4°C until used. The yolk was carefully separated from the white. The resulting IgY antibodies were isolated from the egg yolk, purified through an affinity column[8,‡] and tested by enzyme linked immunosorbent assay (ELISA)[9,§] by antibody binding to solid-phase bound **BSA-2** antigen (Fig. 3). The presence of immunoglobulins **ABY-KLH-2** specific to the hapten **2** or part of it, in the egg yolk was confirmed by indirect ELISA[§] (Fig. 4, ■).

There title was rather low (by a factor of 5) compared to the related **ABM-KLH-2** sera of mice which were immunized by the same conjugate (Fig. 3, compare ■ to ▲)[1] but the total amount of immunoglobulins produced was nevertheless by far, largely superior.

We also observed, using an inhibition ELISA,[¶] that the β-ketoalcohol (1*R*)-**4** did not inhibit the fixation of **ABY-KHL-2** antibodies to the **BSA-2** antigen coated on the plate (IC_{50}:>500 μM) and this is also the case of (i) the *O*-unsubstituted oxime **6a**, which possesses all the functional groups present on the hapten (Fig. 4, ◆ and ★).

Alkoximinoalcohols (1*R*)-**6** (**6b** R = Et, **6c** R = *n*-Bu, **6d** R = *n*-Hex, **6e** R = $(CH_2)_4$–CO_2Me; Fig. 2) which possess the bicyclic structure as well as a side chain related to the linker bind quite efficiently (Fig. 4). Recognition proved to be the best for **6c** (R = Bu, IC_{50}: 16 μM, ●) which probably possess a side chain of the critical length and lower (IC_{50}: 50 μM) when the side chain length decreases (**6b** R = Et, *) or increases **6d** (R = Hex, +).

Furthermore a polar group, such as a carbomethoxy group, on the side chain of sufficient length has little effect on the inhibition (**6e**, IC_{50}: 50 μM) and neither **6c′**, the diastereoisomer of **6c** bearing a hydroxy group in *endo*-position, nor **7** missing the *exo*- or *endo*-methyl group are recognized by the antibodies **ABY-KLH-2** (IC_{50}>500 μM).

We have previously reported[1] similar behavior of **ABM-KHL-2** and **ABM-OVA-1** antibodies derived from mice towards substrates **6a**–**e**, **6c′** and **7** (Fig. 2),[1] whose structures are somewhat related to that of the β-ketoalcohol (1*R*)-**4**.

In conclusion, we have reported that antibodies harvested from hens (IgY) possess a recognition aptitude, for various conjugates, very closely related to antibodies harvested in a different organism (mice, IgG). All these antibodies proved to be highly specific to the whole structure of the hapten including the side chain. This is quite remarkable and also quite unusual.

We are pursuing our work to understand the reasons behind this unusual behavior. Nevertheless, we have clearly shown that chickens are a valuable substitute to mammals for antibodies production.

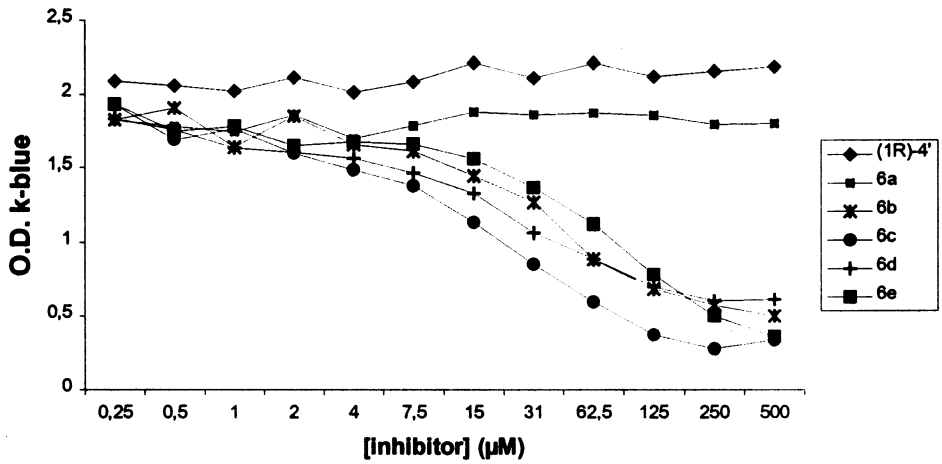


Figure 4. ELISA involving IgY collected from KLH-2 and (1*R*)-**4** and **6a**–**d**.

‡ Preparation of the affinity gel:[8]

Activation step: Divinylsulphone (2 ml) is added to the well stirred suspension of thoroughly washed Sepharose 4B-CL (20 g). After reaction (20°C, 2 h) the resulting gel is collected on a sintered-glass filter and washed with water. The activated gel is stored in water at 4°C until use. *Coupling*: 20 g of vinylsulphone-activated Sepharose, pre-washed with an aqueous solution of sodium carbonate (0.5 M), is mixed to a solution of 2-mercaptonicotinic acid (1.5 mmol) dissolved in aqueous solution of NaOH (0.5 M, 20 ml). The mixture was shaken (20°C, 1 h), glycerol (5 ml) was added and after 6 h the resulting gel slurry was transferred to a glass filter, washed with water and suspended in sodium phosphate buffer solution (25 mM, pH 7.4) before packing.

§ *General procedure for the indirect ELISA*:[9] BSA-2-conjugate (1 μg/ml) is coated (4°C, 24 h) on microtiter plates 96 wells. After blocking unoccupied adsorption sites on the polymer surface, IgY serum is added in various dilutions to the wells and incubated (1 h, 37°C). The wells are washed and rabbit anti-chicken IgG labeled to horseradish peroxidase 1:1000 is used as the secondary antibody. Color development ('key blue' reagent) is stopped after 0.2 h by adding an H_3PO_4 aqueous solution to the wells, and the absorbance is read at 450–630 nm.

¶ *General procedure for the inhibition ELISA*:[9] The procedure is the same as described for the ELISA except that increasing concentration of inhibitors is mixed to the antibodies samples before incubation.

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