IgY Antibodies as Secondary Reagent in FACS Analysis

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Summary
Avian vitellin antibodies (IgY) obtained after immunisation with mouse IgG and mouse IgM in the presence or absence of Freund's complete adjuvant are suited for labelling with FITC or biotin. In FACS analysis the antibodies prove to be excellent tools in detecting mouse monoclonal antibodies of different isotypes.
The working dilution (titre of IgY-FITC) is highest when egg preparations of the secondary and tertiary immune response were used. Double staining of two different antigens can be carried out with direct labelled mab (FITC) and indirect labelled mab by IgY-conjugates (biotin). The two antigens can also be detected by double staining of different isotypes (mabs) by hen anti-mouse IgM FITC and hen anti-mouse IgG biotin-streptavidin-phycoerythrin. The staining obtained with IgY is at least as good or even better than that gained with mammalian secondary antibodies.

Zusammenfassung: IgY als Sekundär-Antikörper bei der FACS Analyse.
Aviäre vitelline Antikörper (IgY), die nach Immunisierung mit Maus-IgG bzw. Maus-IgM mit und ohne Gabe von Adjuvans gewonnen wurden, sind für die Markierung mit FITC oder Biotin geeignet. Mit diesen gekoppelten Antikörpern sind monoklonale Antikörper der Maus unterschiedlichen Isotyps mittels FACS-Analyse nachweisbar. Die Gebrauchsverdünnung (Titer der IgY-FITC-Konjugate) ist dann am höchsten, wenn Eipräparationen aus der sekundären oder tertiären Phase der Immunantwort eingesetzt werden. Doppelmarkierungen zweier verschiedener Antigene können in Kombination mit direkt markierten monoklonalen Antikörpern (FITC) und indirekt markierten monoklonalen Antikörpern durch IgY-Konjugate (Biotin) ausgeführt werden. Ebenso können zwei verschiedene Antigene durch Verwendung monoklonaler Antikörper unterschiedlichen Isotyps und den Einsatz von Huhn-anti-Maus-IgM-FITC und von Huhn-anti-Maus-IgG-Biotin/Streptavidin-Phycoerythrin nachgewiesen werden. Die Markierung durch IgY ist derjenigen, die durch mammäre sekundäre Antikörper erreicht wird, vergleichbar bzw. überlegen.

Keywords: Avian vitellin antibodies, IgY, labelling, FACS analysis

1 Introduction

To detect a wide variety of primary antibodies in immunological assays antibodies as secondary reagents are used. Usually they are obtained from sera of immunised mammals such as goat, sheep, horse, rabbit, rat and guinea pig after bleeding. These reagents are widely used and they have been shown to be excellent tools in developing difficult immunological methods.

In contrast to mammals up to now aves have never gained much attention as producers of antibodies.

Only lately when discussions have been raised about the maintenance, stress situation and bleeding of animals, avian antibodies as an alternative to serum-derived antibodies have been taken into consideration. These antibodies will only have a chance to be used as alternatives when they equal mammalian antibodies in quality and quantity. Furthermore it has to be demonstrated that a) the method to obtain avian antibodies is fast and simple b) the antibodies can be applied in many different assays and c) they can be labelled with enzymes, fluorescence dye and biotin without loosing their specific binding properties.

Here we show that judged by FACS analysis avian vitellin antibodies are excellent tools to detect mouse monoclonal antibodies of different isotyps. The results are discussed with regard to commercial aspects.

2 Materials and methods

HAM-IgG (hen anti-mouse IgG) and HAM-IgM (hen anti-mouse IgM) from single egg preparations, generated at different phases of the immune response, are used to detect surface structures on human peripheral blood lymphocytes using mouse monoclonal antibodies (mabs). The IgY preparations are isolated by PEG-precipitation (Polson et al., 1980) from eggs of immunised SPF-hens.

Conjugation of IgY with FITC was performed according to Reisher and Orr (1968) and biotinylation was carried out as described by Ey et al. (1978) with minor modifications.

Labelled cells were analysed in a FACScan (Becton/Dickinson, Heidelberg, Germany). 2–4 × 10^5 cells were labelled with 50 µl of monoclonal antibody. After three washes cells were incubated with FITC- or PE-labelled or biotinylated antibodies (SA-PE: stained by streptavidin-phycoerythrin).

In FACS analysis following antibodies were used:

- mouse monoclonal antibodies
  BL-TH4 (CD4; isotyp IgM)
  BL-B40 (CD40; isotyp IgM)
  BL-TP3b (CD3; isotyp IgG)

  BL-TP3b-FITC (direct labelled with FITC)
- secondary antibodies from goat and hen
  goat anti-mouse Ig phycoerythrin conjugate (GAM-PE)

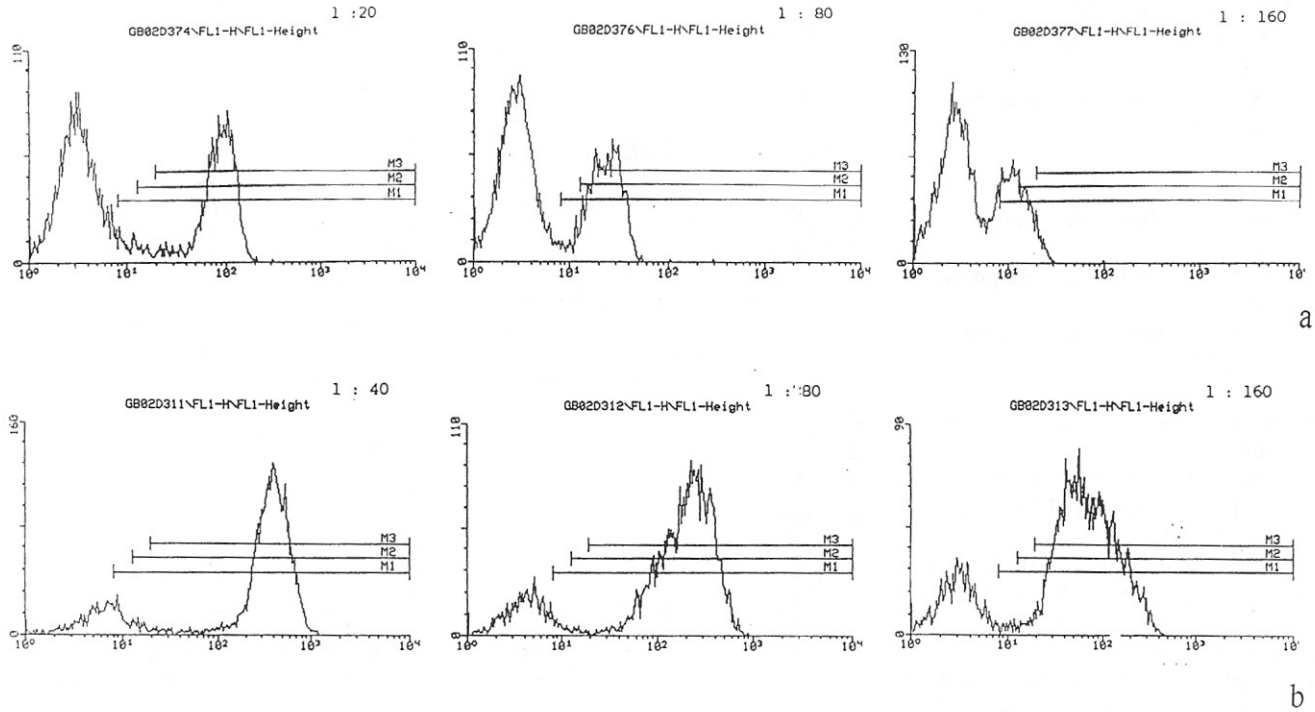

Figure 1: Staining of surface antigens CD3 and CD4 on human PBL with mouse mabs (different isotypes) detected by HAM-IgM-FITC (a: 104-F) and HAM-IgG-FITC (b: 177-F) used in different dilutions.

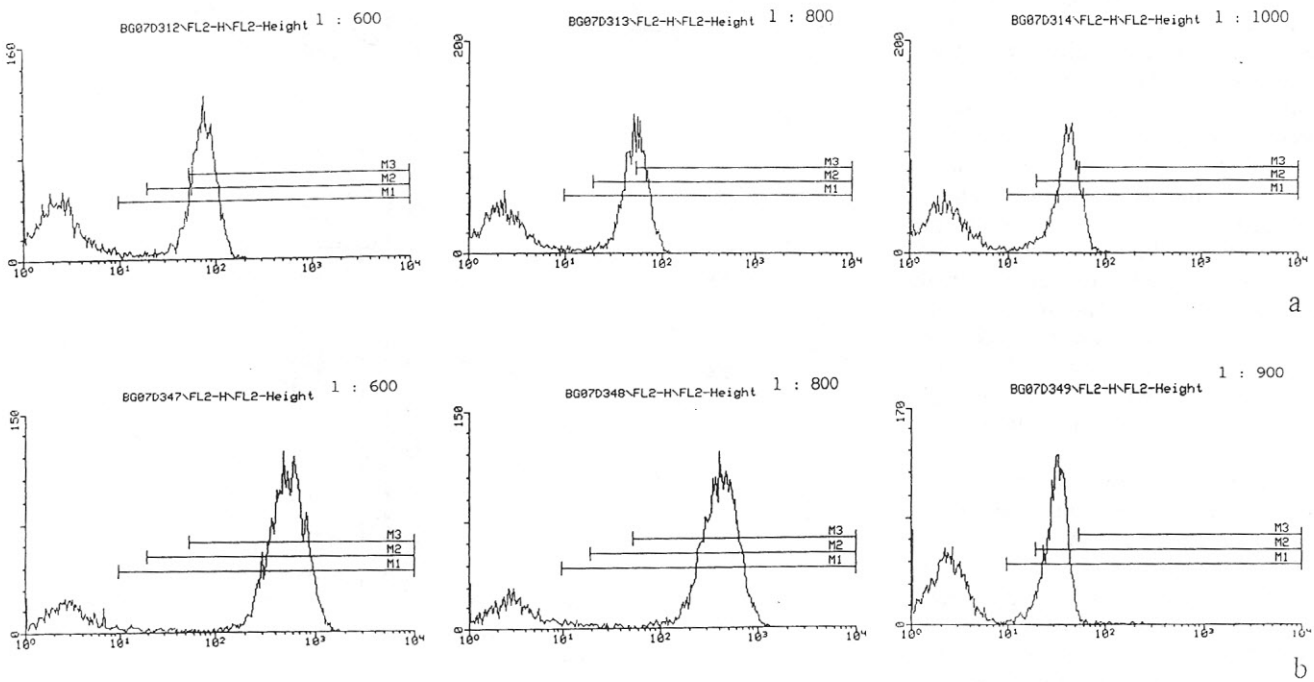

Figure 2: Staining of surface antigens CD3 and CD4 on human PBL with mouse mabs (different isotypes) detected by HAM-IgM-biotin-streptavidin-phycoerythrin (a: 104-B) and HAM-IgG-biotin-streptavidin-phycoerythrin (b: 177-B) used in different dilutions.

hen anti-mouse IgM
104-B (biotinylated probe no. 104)
104-F (FITC-labelled probe no. 104)

hen anti-mouse IgG
177-B (biotinylated probe no. 177)
177-F (FITC-labelled probe no. 177)
121-B (biotinylated probe no. 121)

Staining experiments were carried out as follows:
I Indirect staining of mab with HAM-IgG-FITC, HAM-IgM-FITC, GAM-FITC or GAM-PE,
II Double staining of two different CD antigens with direct labelled mab and indirect labelled mabs by HAM-B-SA-PE or GAM-PE,
III Double staining of two different CD antigens by two indirect HAM-FITC- and HAM-B-SA-PE stained mabs.

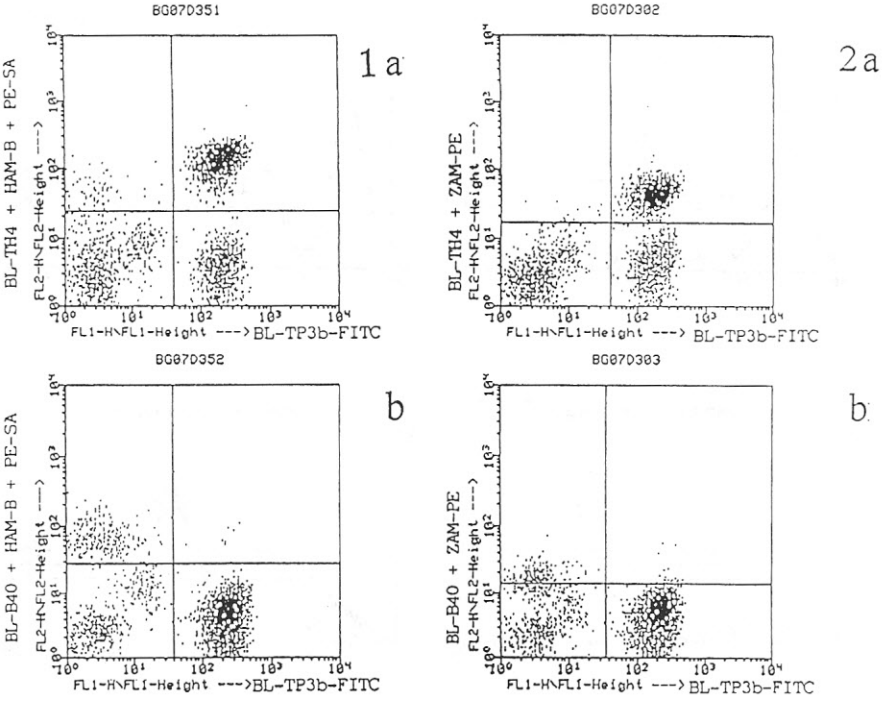

Figure 3: Double staining of human PBL with direct (BL-TP3b-FITC) and indirect labelled mouse mabs detected by HAM-IgM-biotin-streptavidin-phycoerythrin (1a,b: 104-B) or goat anti-mouse Ig phycoerythrin (2a,b: GAM-PE).

3 Results

HAM-IgG and HAM-IgM obtained at different times during immunisation were conjugated with FITC or biotin. Specific binding capacity was tested as described (Behn et al., 1996). For labelling no additional purification steps were necessary. The quality of the conjugates did not so much depend on the coupling method rather than on the content of specific antibodies present in the yolk. The efficiency of the coupling products correlated with the height of the titre. The higher the titre of the starting solution the more effective the coupling products. This stems from the fact, that specific immunoglobulin amounts to a high portion of total IgY.

FACS analysis provides a quick and sensitive method to determine the efficiency of coupling IgY with biotin or FITC. Working dilutions of HAM-FITC and HAM-biotin to label mouse monoclonal antibodies of different isotyps (IgG, IgM) were tested in a standardised system. Working solutions of the single conjugates can be estimated from the histograms (fig. 1). For coupling single preparations were

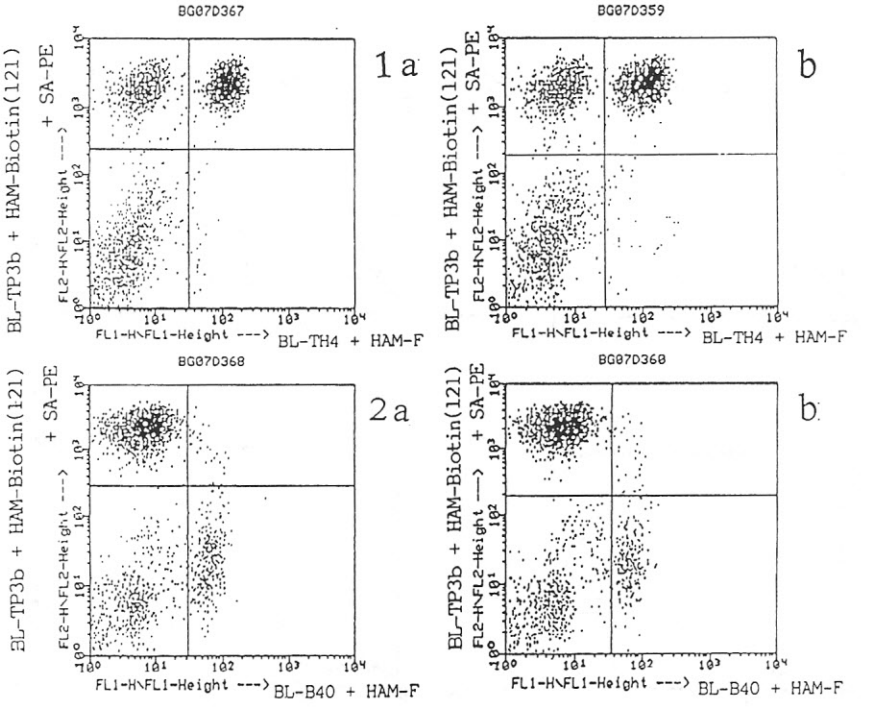

Figure 4: Double staining of human PBL with two indirect labelled mouse mabs (1a,b: BL-TP3b, BL-TH4; 2a,b: BL-TP3b, BL-B40) with HAM-IgM-FITC (104-F) and HAM-IgG-biotin-streptavidin-phycoerythrin (121-B) in the absence (1a 2a) or presence (1b, 2b) of blocking serum.

taken from maxima of the three phases of immune response (PIR, SIR, TIR) taking into consideration the different antigen doses and the mode of application.

Fig. 2 shows biotinylated IgY preparations tested as described above. According to the histograms the preparation no.104-B and the preparation no.177-B should be used in a dilution of 1:1000 and 1:900, respectively.

Double staining experiments of human PBL in which direct labelled mabs are combined with indirect labelled mabs are shown in figure 3. The indirect staining experiments were carried out with avian vitellin antibodies as well as with mammalian antibodies. Dot blots illustrate this suitability of IgY preparations as secondary reagents. In contrast to GAM-PE HAM-IgM-biotin labelled preparations caused a better staining of lymphocyte subpopulations ($CD40^+$ and $CD4^+$, respectively).

With the aid of specific coupling products from hen egg two indirect labelled monoclonal antibodies of different isotypes (IgG and IgM, respectively) could be detected on human PBL (fig. 4). In dot blot analysis different subpopulations were significantly discriminated. The same figures were also obtained without a blockade by normal serum passing the subsequent labelling steps.

Table 1 shows working dilutions of different conjugates obtained from IgY preparations of single eggs. Preparations that originate from hens immunised with either mouse IgM or mouse IgG are compared. It is obvious that dilutions of working solutions of IgY conjugates from both the secondary and tertiary immune response are higher that those from the primary immune response and that adjuvant lead to an increase of the response. The amount of the antigen applied was not that relevant to the outcome of the experiment.

To calculate the greatest possible labelling of IgY data from two separate eggs obtained after the TIR were used. These data indicate how many samples can be labelled by IgY (FITC) and analysed by FACS (table 2).

Table 1: Comparison of different HAM-FITC-conjugates prepared from hen IgY obtained from eggs after different modes of immunisation

Antigen/Adjuvans	IgY-FITC-conjugates (working dilution) PIR	SIR	TIR
Mouse IgM (1mg)			
with FCA	n.d.	1:2000	1:3000
without FCA	1:50	1:200	1:500
Mouse IgM (0.1 mg)			
with FCA	1:80	1:2400	1:2000
without FCA	1:50	1:100	1:100
Mouse IgG (1mg)			
with FCA	1:50	1:1000	1:2000
without FCA	1:20	1:50	1:50
Mouse IgG (0.1mg)			
with FCA	1:10	1:2400	1:2400
without FCA	1:10	1:10	1:20

FCA = Freunds complete Adjuvans, PIR = primary immune response, SIR = secondary immune response, TIR = third immune response

Table 2: Theoretical number of tests which can be carried out in FACS analysis if all IgY from one egg of hens immunized with mouse IgM (egg no. 411) and mouse IgG (egg no. 667), will be labeled with FITC

411 (1.0mg; TIR)	Egg no. (Antigen dose; mode of immunisation)	667 (0.1mg; TIR)
100mg	Amount of IgY after preparation	40mg
1:3000	Working dilution for FACS analysis after labelling with FITC	1:2000
12×10^6	Number of tests which can be carried out with the amount of IgY prepared from one egg	3.2×10^6

3 Discussion

To prove the suitability of avian vitellin antibodies as secondary antibodies we immunised hens with mouse IgM and mouse IgG in the presence or absence of FCA. Standard procedure were used (Reisher and Orr, 1968; Ey et al., 1978) to label PEG precipitations of eggs with FITC or biotin.

IgY can be labelled directly even in the presence of unspecific IgY antibodies without the need of further purification procedures. These labelled preparations proved to be excellent tools as secondary antibodies in FACS analysis. Specific binding of hen anti-mouse IgM or hen anti-mouse IgG was not influenced by the coupling procedure. The quality of the labelled product depends on the immunisation protocol.

Immunogenicity of the antigen and the presence of adjuvant influenced the titre of the egg preparations to a greater extent than the amount of injected antigen. Conjugates prepared from eggs of the primary immune response were less efficient than those from the secondary immune response as to quality and quantity. The quality did not improve much when conjugates were prepared from the tertiary immune response. In FACS analysis the HAM-IgM and HAM-IgG coupled with either FITC or biotin can be used for indirect staining of the anti-CD monoclonal antibodies as secondary antibodies. Compared to goat anti-mouse FITC these labelled antibodies were of equal