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Non-invasive Monitoring of Immunization Progress in Mice *via* IgG from Feces

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Abstract. *A non-invasive method to monitor the humoral immune response in mice after immunization is described. From fecal pellets of an individual mouse, a sufficient amount of active immunoglobulins or their fragments can be extracted to perform a regular examination of the status of the immune response by immunoassay. Hapten-specific antibodies from the feces of mice from three immunization trials showed very similar characteristics to those obtained from serum at a given date. Therefore, it can be suspected that some serum IgG enters the intestinal lumen and ends up in the feces, where they appear to be considerably stable. Hapten-specific IgAs were not found in the feces. Being able to analyze antibody titers in feces could be an interesting animal welfare refinement to standard practice that does not entail repeated blood sampling.*

Monoclonal antibody production in routine use today still has to rely on the immune response of immunized animals (1, 2), although considerable efforts are being made to improve animal protection in antibody production, such as isolating antibodies from egg yolk (3, 4) or milk (5), or by applying *in vitro* techniques putting forward recombinant antibodies (6), antibody libraries (7) and single cell-based techniques (8).

Monitoring of the immune response is not only mandatory to determine the moment of a sufficiently high specific antibody titer but also to observe potential increase of affinity during affinity maturation (9, 10) and to select the 'best' from several mice immunized in parallel as well as identifying non-responders. In the production of antibodies against small molecules (11), this monitoring is a critical step because it is the first possibility to assess the success of earlier process stages, *i.e.* (i) the selection of the hapten used as mimetic of the small molecule; (ii) the modification of such hapten to enable its coupling to a carrier protein; (iii) the selection of the carrier protein as well as (iv) the coupling method and hapten:protein ratio obtained (12, 13). Far from being the Achilles' heel of the process, the *in vivo* monitoring of the immune response in animals has seen modest improvements over the years. As a matter of fact, the estimation of antibody titer and the tracking of affinity maturation (14) between boosts is performed by probing the animal's blood (15). For mice, facial vein puncture, retrobulbary (orbital sinus) venipuncture, tail incision or tail vein venipuncture are practiced. These methods, particularly when the multiple collection of serum samples from the same mouse is needed, require considerable training and experience of the experimenter. Nevertheless, they cause significant stress to the animal and risk of impairment of the mouse's health. Compared to serum retrieval, collection of mouse feces represents several advantages: it is non-invasive, there is no need for needles and syringes, the animal remains unhurt over the immun-ization period and samples can be collected on a daily basis.

Immunoglobulin G (IgG) is the only antibody isotype with an extended half-life (16) and most monoclonal antibodies represent this isotype. It is produced at different anatomic sites such as the spleen, lymph nodes, lymph tissues, *etc*. Its presence in the feces, together with IgA (so-called 'coproantibodies'), has been known for almost 90 years (17-19), but the question concerning its origin(s) remains an active research field (20-22). The local production of IgG by the intestinal mucosa has been evidenced by both *in vivo* and *in vitro* experiments (23, 24), while the shuttle mechanism of circulating serum IgG into the intestine has received

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somewhat less attention (25-27) and just recently started to be elucidated (28-31). The major histocompatibility complex class I-like neonatal Fc receptor (FcRn) seems to play a role in transporting circulating IgG throughout the intestine endothelium (transcytosis) as well as in reverse, recycling IgG and IgG–antigen complexes (22, 32). Indeed the FcRn receptor is a key homeostatic regulator for IgG, controlling its transport (bidirectional) across several epithelial barriers in mammals to affect both systemic and mucosal immunity (22, 23, 33, 34). The role of FcRn, which was discovered in the late 1980s (35), in IgG homeostasis regulation was comprehensibly reviewed recently (16, 34). The studies of Besser *et al*. (25) demonstrated that around 68% of circulating IgG1 clearance occurrs by transfer to the gastrointestinal tract in calves using radioactively labeled (^{125}I) IgG administered intravenously. Externest *et al*. (36) described a correlation of serum IgG concentrations with IgA in mouse feces but not with fecal IgG. In humans, significant quantities of IgG have been found to be secreted into the intestinal lumen (21).

Here we describe observations during the monitoring of three independent *in vivo* immunizations for three unrelated haptens: isolithocholic acid (ILA), ochratoxin A (OTA) and triacetone triperoxide (TATP). In the feces of intraperitoneally immunized mice we found antigen-specific IgG was able to bind the hapten with similar affinity as the IgG from serum samples of these mice. ILA is a bile acid formed from 3-keto-5β-cholanoic acid reduction (37); OTA is a mycotoxin produced by some *Aspergillus* and *Penicillium* species (38) and is known to be found in some commodities such as cereals, red wine, coffee and dried fruits (39); TATP is an improvised explosive which is highly attractive to terrorists due to its easy and cost-efficient synthesis and lack of detection by the conventional surveillance methods in use (40).

Materials and Methods

Antibodies. Polyclonal anti-IgG sera were obtained from Acris Antibodies (Herford, Germany): anti-mouse IgG (H&L) whole molecule, from sheep (R1256P, Lot 20243, 2.2 mg/ml); anti-mouse IgG - F(c), from goat (R1612P, Lot 22712, 3.7 mg/ml). Polyclonal anti-IgA mouse serum (from goat, NB 7501, Lot A23; 1 mg/ml) was obtained from Novus Biologicals *via* Acris Antibodies.

Immunogens and tracer synthesis. Horseradish peroxidase (HRP), EIA grade, was obtained from Roche (Mannheim, Germany). Guardian™ (peroxidase conjugate stabilizer/diluent) was purchased from Thermo Scientific (Perbio, Bonn, Germany). Bovine serum albumin (BSA), fraction V, receptor grade, was from Serva (Heidelberg, Germany). *N,N*-Dimethylformamide (puriss.), *N,N'*-dicyclohexylcarbodiimide (puriss.), *N*-hydroxysuccinimide (purum) and tetrahydrofuran were from Fluka (Neu-Ulm, Germany). ILA (5β-cholanic acid-3β-ol), melting point 181-182.5˚C, was from Steraloids Inc. (London, UK). OTA was purchased from Alexis Biochemicals (Enzo Life Sciences, Lörrach, Germany). TATP was synthesized according to Wolffenstein (41) and recrystallized three times from hot methanol (42). Acetone ('picograde'), was obtained from LGC Promochem (Wesel, Germany). PD 10 columns containing Sephadex G 25 were obtained from GE Healthcare (München, Germany). Polypropylene tubes (1.5 ml) were purchased from Eppendorf (Hamburg, Germany).

Protease inhibitors. Aprotinin from bovine lung and leupeptin hydrochloride were purchased from Sigma-Aldrich (München, Germany), Pefabloc® SC (4-2-aminoethyl)benzenesulfonylfluoride hydrochloride) and Complete Protease Inhibitor Cocktail tablets were from Roche (Mannheim, Germany); and Pepstatin A was from Applichem (Darmstadt, Germany).

Enzyme-linked immunosorbent assay (ELISA) reagents. Sodium phosphate dibasic dihydrate, sodium phosphate monobasic dihydrate, potassium phosphate monobasic, potassium dihydrogen citrate, sodium chloride, sodium carbonate and sodium bicarbonate were of Fluka 'ultra' quality and obtained from Sigma-Aldrich (München, Germany). Trifluoroacetic acid (for protein sequence analysis) and calcium nitrate tetrahydrate (ACS) were purchased from Merck (Darmstadt, Germany). Research grade 3,3',5,5'-tetramethylbenzidine (TMB) and pure Tween™ 20 were from Serva (Heidelberg, Germany). Phosphoric acid (85%), *N,N*-dimethylacetamide (puriss.) and sodium azide (>99%) were supplied by Sigma-Aldrich (München, Germany). Hydrogen peroxide 30% Trace select® and calcium sulfate dehydrate (puriss.) were from Fluka. Sulfuric acid (95-97%) and hydrochloric acid (32%), were of Baker analyzed grade (Mallinckrodt Baker, Griesheim); methanol, 2-propanol and acetonitrile were HPLC grade (Mallinckrodt Baker, Griesheim). Ultrapure reagent water (hereafter referred to as Milli-Q water) was obtained by running demineralized water (by ion exchange) through a Milli-Q® water purification system (Millipore Synthesis A 10; Millipore, Schwalbach, Germany). Transparent microtiter plates with 96 flat-bottom wells possessing high protein-binding capacity (MaxiSorp™) were purchased from Nunc (Thermo Scientific).

Animals. Female, three-month-old BALB/c mice were bred and housed at the Biotechnology Department of Potsdam University, Golm, Germany (for ILA and OTA immunizations) and at Charles River Laboratories, Sulzfeld, Germany (for TATP immunization). The studies had approval of the respective Animal Care and Use Committees at the institutions.

Preparation of the immunogens and tracers. The haptens ILA, a spacer derivative of TATP with a carboxy terminus described by Walter *et al*. (42) and an OTA derivative carrying a triglycine spacer were coupled to the carrier protein BSA *via N*-hydroxysuccinimide chemistry with carbodiimide to produce the immunogens according to Tatake *et al*. (43). The same method was used to synthesize the respective enzyme tracers, using HRP (44). ILA and the TATP hapten were coupled directly to the proteins, while for OTA, a triglycine spacer was used to link it to the protein. The activated esters were obtained in anhydrous *N,N*-dimethylformamide, except the TATP hapten, which was produced in anhydrous tetrahydrofuran. Some fractions of the immunogens were precipitated using acetonitrile.

Immunizations. For each hapten, three BALB/c mice were immunized intraperitoneally using 20 or 50 μg of the insolubilized immunogen in Freund's complete adjuvant supplied by Difco

(Lawrence, KS, USA). The animals were boosted regularly (at 4 to 6-week intervals) with the (soluble) immunogen (10-50 μg) in PBS buffer. Serum samples (80-100 μl, retrobulbary puncture) were taken five to seven days after each boost to evaluate the titer and affinity maturation of the hapten-specific antibodies. After leaving the blood sample for 1-2 hours at room temperature, the samples were centrifuged (3,000 × *g* for 5 minutes) and the supernatant recovered, to which *ca*. 10% (v/v) of a 0.1% (w/v) azide solution was added. Serum samples were then stored at 4˚C.

Feces were collected 30, 42 and 18 weeks after the first immunization in the case of ILA, TATP and OTA, respectively, together with serum samples of the same day. For TATP and OTA, the mice were placed in separate cages for one day for fecal sampling (up to 1 g per animal) and serum collection. For ILA, the three immunized mice were sampled together for feces (mixed sample). Husbandry and handling of animals, immunization and blood sampling/feces collection were performed at the facilities of Potsdam University at Golm, Germany, and at Charles River Laboratories, Kißlegg, Germany.

Fecal extraction. A slightly different protocol is described for each hapten because the three immunizations were performed independently and at different times by different operators. The protocols were based on previous extraction methods described by Dion *et al*. (45). The final dilution factors given for serum samples and fecal extracts result from serial dilution experiments in order to obtain comparable optical densities in the ELISA measurements.

Feces from ILA-BSA-immunized mice were extracted using PBS buffer (pH 7.6) containing 1% (w/v) sodium azide supplemented with Complete Protease Inhibitor Cocktail tablets from Roche (1 mini-tablet, EDTA-free, per 10 ml buffer). One gram of air-dried feces was shaken overnight (20 hours) with 10 ml of the aforementioned buffer. The extract was aliquoted into 2 ml Eppendorf tubes which were thereafter centrifuged at 15˚C, 15,000 × g for 5 minutes. The supernatant was diluted 10-fold in PBS buffer prior to performing the ELISA.

Feces from TATP-BSA-immunized mice were extracted using PBS buffer (pH 7.6) containing 1% (w/v) sodium azide, 1% (w/v) BSA (or 0.1% (v/v) Tween 20) and protease inhibitor mix (0.3 μM aprotinin from bovine lung, 4.3 μM leupeptin hydrochloride, 8.4 μM Pefabloc® SC and 365 μM Pepstatin A). Air-dried feces (500 mg) were shaken overnight (23 hours) with 7.5 ml of the described buffer. The extract was aliquoted into 2 ml Eppendorf tubes which were thereafter centrifuged twice at room temperature, 14,000 × *g* for 10 minutes. The supernatant was not diluted prior to performing the ELISA.

Feces from OTA-BSA-immunized mice were extracted using PBS buffer (pH 7.6) containing 1% (w/v) sodium azide, 3.5% (w/v) BSA and a protease inhibitor mix (0.3 μM aprotinin from bovine lung, 4.3 μM leupeptin hydrochloride, 1 μM Pefabloc® SC and 3 μM Pepstatin A). Air-dried feces (500 mg) were shaken overnight (20 hours) with 10 ml of the described buffer. The extract was centrifuged twice at 4˚C, 14,000 × *g* for 15 min. The supernatant was diluted 10-fold in PBS buffer prior to performing the ELISA.

ELISA. All the assays were performed in MaxiSorp plates using PBS buffer (pH 7.6). The respective capture antibody (anti-IgG whole molecule; anti-IgG Fc or anti-IgA) was diluted to 1 μg/ml for all the assays. The serum samples were diluted in PBS containing 1% (w/v) BSA at 1:100,000 anti-ILA; 1:10,000 anti-TATP; and 1:80,000 anti-OTA.The standard solutions were prepared in Milli-Q water, with the exception of the stock solutions which were prepared in an appropriate organic solvent (2-propanol for ILA, methanol for TATP and OTA). The enzyme tracers were diluted in PBS containing 1% (w/v) BSA (1:100,000 for ILA, 1:10,000 for TATP) or PBS containing 0.1% casein (1:10,000 for OTA). Calibration was performed using serial dilutions of stock solutions of the target analytes in Milli-Q water. Optical density readouts were interpolated by a logistic 4-parameter function (44) the 'C-value' representing the inflection point of the sigmoidal curve which is similar to 50% inhibitory concentration (IC_{50}).

Results and Discussion

Hapten inhibition: feces vs serum. The fecal extracts provide similar inhibition curves (*i.e*. comparable shape and C-values) to those obtained with the sera of immunized mice sera for all three immunizations, as shown in Figure 1A-C. Dilutions of sera and fecal extracts had to be individually adapted to provide a comparable maximum OD (A-value, 'blank' OD). The fecal curve of the ILA-BSA immunized animals (Figure 1A) depicts results from a mixed fecal sample of the three animals; the displayed serum curves for the other immunizations originate from one single mouse since no significant differences in their C-values were found between the animals besides antibody titer.

Capture antibody specificity: whole molecule (WM) vs Fc. Capture antibodies of different specificity have been used for the ELISA, one of them from an immunization with an 'WM' mouse IgG, the other from immunization with the Fc (crystallizable fragment) of mouse IgG.

Binding to the anti-WM- and the anti-Fc-specific capture antibodies seems to be dissimilar for feces and sera, respectively. As Figure 2A shows, for fecal extracts, higher signals were obtained when anti-WM was used, while for the sera, the higher signal was obtained with the anti-Fc antibody (Figure 2B). The experiment was repeated three times with different mouse sera and mouse fecal extracts and the same outcome was observed. The coating/capture antibodies were always applied at the same concentration and the assays performed within the same microtiter plate. In the examination of the three mice immunized with the TATP-BSA conjugate, in the indirect ELISA all three mice showed specific antibody titers but in the direct ELISA format, just one mouse showed a specific titer. Exactly the same pattern was obtained from the fecal samples.

We were not able to prove unambiguously if the binding observed might not also be due to antibody fragments and/or degradation products. In future trials, this could be elucidated by gel electrophoresis and Western blot if sufficient amounts of protein are isolated.

Hapten-specific fecal IgA vs fecal IgG. Hapten-specific IgA was not found in any of the immunized mice. The results are

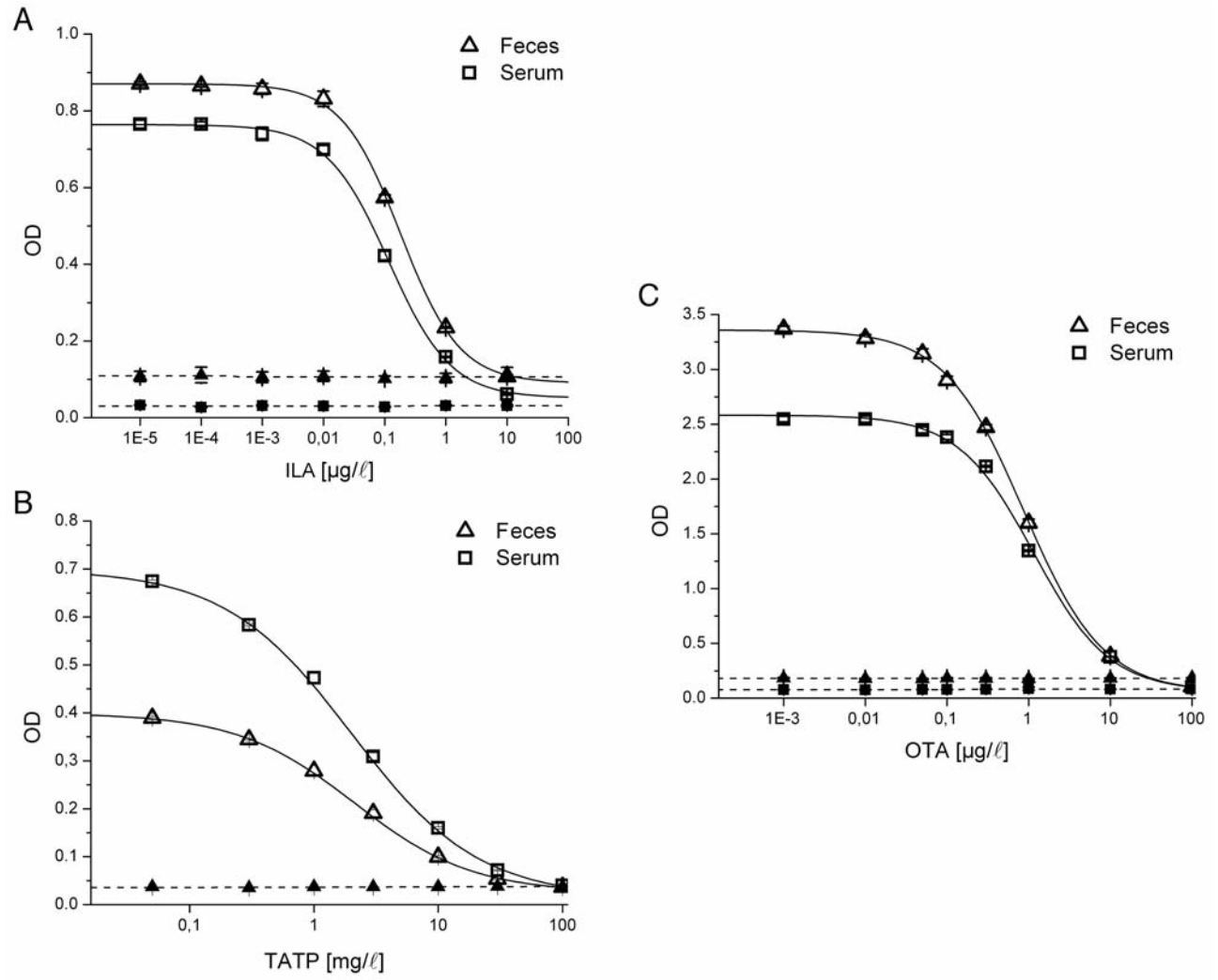

Figure 1. *Calibration curves obtained from mouse sera and feces. Sera and feces were taken from immunized (open symbols) and non-immunized mice (closed symbols) the same period after immunization, which was 30 weeks for isolithocholic acid (ILA), 42 weeks for triacetone triperoxide (TATP) and 18 weeks for ochratoxin A (OTA), respectively. The solid lines represent the calibration curves for the different haptens: A: ILA with C-value of 0.17 µg/l (feces) and 0.12 µg/l (serum); B: TATP with C-value of 2.1 mg/l (feces) and 2.0 mg/l (serum); C: OTA with C-value of 0.25 µg/l (feces) and 0.17 µg/l (serum). Error bars: standard deviation of triplicate determinations.*

illustrated exemplarily with the ILA immunization shown in Figure 3. The microtiter plate was coated partly with anti-mouse IgA and partly with anti-mouse IgG (WM). Only hapten-specific IgG was detected in the feces. Moreover, non-specific IgA was present in high concentration in the feces as illustrated by the high background in Figure 3. The same test was performed for the sera and the same outcome was observed except for a marked difference in the IgA background: the OD values from sera were much lower and constant at 0.012 (±0.002).

Considerations regarding the fecal extraction buffer. Feces from ILA-immunized mice were extracted with the described buffers (see Materials and Methods section) as well as with PBS buffer (without protease inhibitors) and even Milli-Q water. Although we could not compare the IgG titers because the feces were collected at different times, the curve shapes and the C-values were highly comparable regardless of the extraction solution used. Furthermore, a Milli-Q water extract stored at 4˚C was reanalyzed 6 months later and gave the same results (data not shown).

Feces from TATP-immunized mice were extracted with the described buffer containing either BSA or Tween 20 and no differences were found between the calibration curves. Furthermore, the use of protease inhibitor mix as proposed in literature (46) is not necessary to protect IgG from degradation. Antibodies appeared to be quite stable in air-

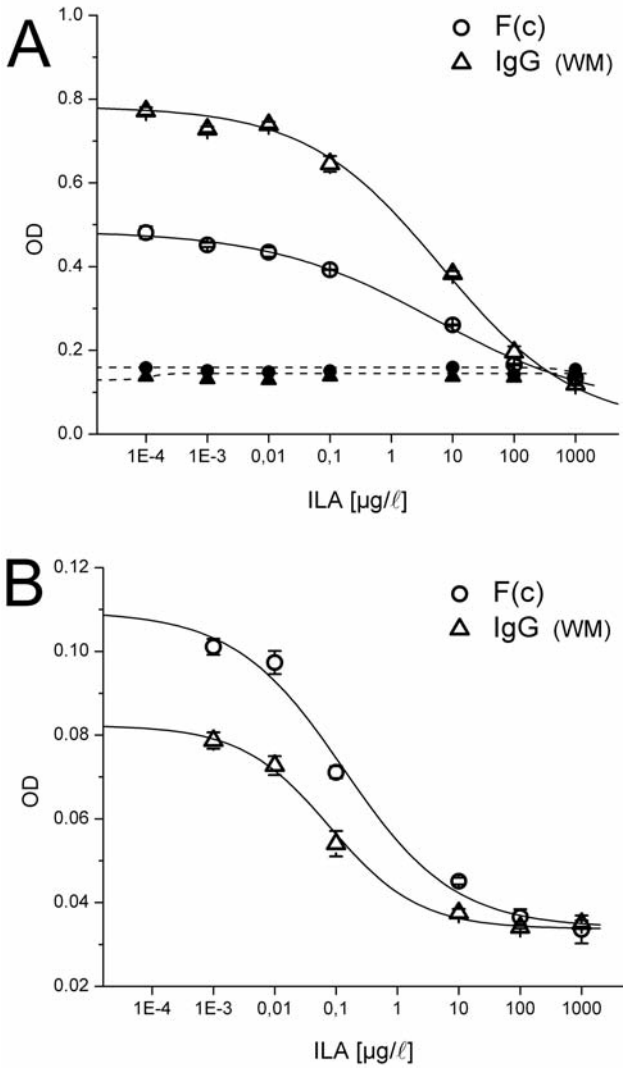

Figure 2. *Isolithocholic acid (ILA) calibration curves using different anti-IgG coating. The comparison between two coating antibodies is shown: anti-mouse IgG 'whole molecule' (WM) and anti-mouse IgG Fc. The open symbols represent immunized animals and the closed symbols non-immunized ones. A: Fecal extracts; B: serum. Means and standard deviations (error bars) of results from 3 different sera and 3 different fecal extracts are shown. Serum and fecal samples were collected 30 weeks after the initial immunization.*

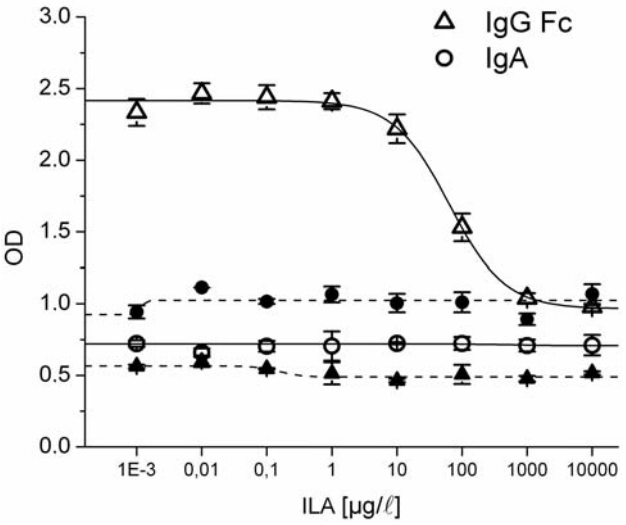

Figure 3. *Isolithocholic acid (ILA) calibration curves using anti-IgG and anti-IgA coating, respectively. Calibration curves obtained using fecal extracts and two different coating antibodies: anti-mouse IgG Fc and anti-mouse IgA. The open symbols represent feces from immunized mice, while the closed symbols are from non-immunized ones. Error bars: standard deviation of triplicate results. Serum and fecal samples were collected 30 weeks after the initial immunization.*

dried samples of feces, a finding reported before for IgA (47). This facilitates transport and storage of samples.

Application of the method for Mab production. We found that the immune response in an individual mouse can be monitored non-invasively on the basis of sampling and extraction of the animal's feces. Aqueous extracts of mouse feces provided stable and equivalent results to those obtained from serum samples, except for the absolute IgG titer, which was considerably lower in the fecal extracts, requiring smaller dilution factors. As a cause, it can be suspected that some serum IgG enters the intestinal lumen and ends up in the feces.

No selective IgA was found in the feces. Yet the final proof that coproantibodies are reliable indicators of splenic B-cell antibodies (which will be used for the fusion) still has to be provided. Assaying salivary antibodies that are also proposed for this purpose (48) involves much more difficult procedures in sampling.

For future studies several improvements should be pursued, an adopted housing system for individual mouse probing and optimized storage and extraction protocols for the feces to increase the sensitivity of the analysis. Moreover, a method of standardizing samples using total antibody concentration or protein content could be helpful to reduce potential variations between animals and samplings. Tracing the immune response of mice which are immunized in order to produce monoclonal antibodies is a very frequent task in immunochemical laboratories. Monitoring is necessary to select the most reactive mouse (or rank different mice) and to determine when peak titers have been reached. This level of comparison, *e.g.* wether the titers from feces also rise and plateau over time, was not possible in our trials. Adopting the novel approach would represent considerable progress as it permits early and frequent testing for the development of specific antibody titer in the several animals usually immunized in parallel, thus saving time. Moreover, the technique would allow those less experienced in animal handling to perform the studies. Collecting feces instead of sampling blood allows for a daily

monitoring of the immunization progress in mice without hurting or even touching the animals, so that in contrast to serum sampling, mice experience much less distress. In the 3R concept (replacement, reduction, refinement) of animal protection (49, 50), this represents a welfare 'refinement' to standard practice, with the potential of improving laboratory animal welfare considerably.

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