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Development of monoclonal antibodies specific for *Ricinus* agglutinins

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Ricin is a highly toxic, dichain ribosome-inactivating protein present in the seeds of *Ricinus communis* (castor), grown principally as a source of high quality industrial lubricant and as an ornamental. Because of its presence in industrial byproducts and its documented use for intentional poisoning, there is a need for analytical methodology to quantify ricin in both castor extracts and food matrices. We developed a panel of monoclonal antibodies to ricin, with most having strong cross-reactivity with RCA-1, a homologous but less toxic castor agglutinin. Some of the IgM-producing hybridomas appeared to produce a second, IgG isotype and were further analysed by fluorescence-activated cell sorting. The antibodies were effective in various ELISA formats, many with IC₅₀'s in the range of 0.1–10 ng/mL and minimal matrix effects in skim milk. Assay specificity can be adjusted for analytical needs by varying the combination of antibodies in a sandwich ELISA format.

Keywords: ricin; *Ricinus communis* agglutinin; castor; monoclonal antibody

Introduction

Ricin is a protein synthesised in the castor plant, *Ricinus communis*, and found primarily in the seeds, colloquially known as beans (reviewed by Lord & Lynne, 2005). The toxin is a member of the Type II ribosome-inactivating proteins (RIPs), dichain or A/B toxins, and is an N-glycosidase that initiates depurination and cleavage of 28S ribosomal RNA at position 4324. The cleaved RNA is not able to bind Elongation Factor 2, needed for protein synthesis, and the ultimate result is cell death. The two chains have molecular weights of about 32,000 and are bonded by a single disulfide. The A chain (RTA) is a highly active N-glycosidase, inactivating more than 1500 ribosomes per minute. The B chain (RTB) binds to galactosamine or N-acetylglucosamine surface receptors and enters the cell via receptor-mediated endocytosis. Efficient cellular uptake of the toxin and the high turnover number of the glycosidase contribute to its toxicity.

In its crude form, ricin is often found in association with a lectin, *Ricinus communis* agglutinin-1 (RCA-1), having hemagglutinating activity. The RCA-1 tetramer contains two non-covalently bonded ricin-like dimers, but with greatly reduced toxicity compared to ricin. The transcripts for ricin and RCA-1 are too similar to distinguish by Northern blot analysis, but have recently been distinguished

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by a reverse transcription-polymerase chain reaction method developed at this Center by Chen, He, and McKeon (2005). Cross-reactivity of anti-ricin antibodies with RCA-1 is a problem in some immunoassays, including commercialised methods, depending on which epitopes of ricin are detected.

Ricin toxicology has been summarised by Doan (2004). This protein is most toxic through inhalational exposure, and it is estimated that about 35 µg of ricin would cause mild illness and 200 µg would be lethal to humans. Ricin is absorbed from the gastrointestinal tract and transported to vital organs, including the spleen and liver (Ishiguro, Tanabe, Matori, & Sakakibara, 1992). It is ironic that this biothreat agent can be fashioned into a specific, effective therapeutic weapon against cancer cells by conjugating the A chain to an appropriate antibody, first demonstrated by Krolick, Villemez, Isakson, Uhr, and Vitetta (1980). Much information about the immunochemistry of ricin was derived from experiments to optimise the performance of such immunoconjugates, and has been summarised in Maddaloni et al. (2004). The parenteral lethal dose for ricin has been reported to be between 5–36 µg kg⁻¹ in the mouse (Madsen, 2001; Zhan & Zhou, 2003), with the oral toxic dose approximately 400-fold lower (Madsen, 2001). The minimum lethal human oral dose is estimated at less than 1 mg, equivalent to about one castor bean.

A number of rapid immunologically based assays have been developed for ricin, including immunoassays (Poli, Rivera, Hewetson, & Merrill, 1994; Shyu, Chiao, Liu, & Tang, 2002), activity assays (Hale, 2001), immunochromatographic devices (R. Shyu, H. Shyu, Liu, & Tang, 2002), and immunosensor prototypes (Stine, Pishko, & Schengrund, 2005). A review of related methodology (Garber, Eppley, Stack, McLaughlin, & Park, 2005) highlighted the significant issues of cross-reactivity and a “hook effect” in some assays, including in relatively simple liquid matrices. The hook effect limits rapid screening because it necessitates a dilution series for each sample to be tested. Hale (2001) developed a rapid luminescence assay for RIPs, using the cell-free translation of luciferase mRNA as detection principle. By performing the assay in the presence and absence of a suitable ricin-inhibiting antibody, ricin could be measured specifically. A drawback of the method is that it yields an analytical result as the difference between two determinations, inherently subject to greater experimental error than direct measurements. In addition, the assay does not readily distinguish the activity of the intact toxin from that of the isolated A chain, which requires a functional B chain to gain entrance to whole cells. This assay has recently been used to estimate the stability of ricin in food matrices (He, Lu, Cheng, Rasooly, & Carter, 2008). In addition to these *in vitro* assays, mass spectrometric methods for ricin have been developed (Fredriksson et al., 2005; Hines, Brueggemann, & Hale, 2004; Na et al., 2004), and several assays have been commercialised (see Garber et al., 2005; Garber, 2008). Because performance data in complex food matrices is generally lacking and because of our own observations of unacceptable performance of some commercialised methods (unpublished observations), we prepared a panel of mouse monoclonal antibodies (mAbs) to ricin and analysed their performance in sandwich ELISA format. A number of apparent dual-isotype hybridomas were identified. These were analysed by fluorescence-activated cell sorting (FACS), and the secreted antibodies were studied by affinity chromatography and ELISA.

Materials and methods

Toxins

Ricin, the isolated A and B chains, and RCA-1 were obtained from Vector Laboratories (Burlingame, CA, USA).

Toxin-specific antibodies

The following commercial polyclonal detection antibodies were used in these studies: Rabbit anti-ricin (R1254, Sigma-Aldrich, St. Louis, MO, USA), biotinylated goat anti-ricin (BA-0084, Vector). mAbs were produced as follows. BALB/c mice were inoculated 2–4 times, at approximately 3-week intervals intraperitoneally with 25 µg of immunogen as an oil-in-water emulsion with metabolisable adjuvant (S6322, Sigma). Because of the toxicity of RCA-1, antibodies to this agglutinin were elicited in mice previously inoculated (three times) with RTA and then twice with RCA-1. Immunogens were administered intravenously in saline 3–4 days cell before fusion. Spleen cells were fused with P3-X65-Ag8.653 myeloma cells using polyethyleneglycol. Aminopterin-resistant hybridomas were selected and screened by direct ELISA on antigen-coated microwell plates and cloned by limiting dilution. Antibodies were produced *in vitro* by growing cells in T-flasks with CCM-1 medium (HyClone, Logan, UT, USA) containing up to 1% fetal bovine serum (depending on cell line requirements) or by “weaning” hybridomas from high-serum medium, to achieve a dense culture in RPMI-1640 + 1–2% fetal bovine serum. mAbs were used as culture supernatants, except for mAb 1506, purified by affinity chromatography on a recombinant Protein G-agarose column (MAb-Trap, GE Healthcare, Piscataway, NJ, USA).

ELISA methods

Preparation of assay plates

Solid plates or arrays of 2 × 8 wells (Immulon II, Dynex, Chantilly, VA, USA) were filled with 100 µL of 5 µg/ml protein solution (ricin, related proteins, or “capture” antibody IgG) in phosphate-buffered saline ([PBS], 5 mM sodium phosphate, 150 mM NaCl, pH 7.0) and incubated, with shaking, for 4 h. Plates were rinsed five times with distilled water; potentially sticky sites were blocked by filling wells with 200 µl PBS containing 10 mg/ml BSA, 0.02% Tween[®]-20, and incubating 1 h, with shaking. Following rinsing, as above, wells were filled, with 200 µl of 2% sucrose solution. Following incubation for 30 min, wells were emptied and dried at 37°C, and plates were stored, desiccated at 4°C, for up to three months.

Assay procedures

Incubations were routinely conducted at room temperature for 30 min, with washing (distilled water, five times) between steps. To minimise antibody binding dependent on lectin interactions with the B chain of ricin or agglutinin, a mixture of PBS-BSA-Tween[®]-20 and 0.5 M galactose (4:1) was used as diluent, unless noted. Mouse antibodies (serum antibodies and mAbs) were detected with 100 µl/well, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (0.1 µg/ml, #81-6720, Zymed Laboratories, S. San Francisco, CA, USA). For sandwich ELISA assays using mAbs

in the form of culture supernatants, ELISA wells were coated with goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL, USA), prepared as described above. Just before assay, mAbs were applied to the wells (approximately 1 µg/ml IgG, purified or as culture supernatant) and incubated for 30 min. Plates were washed and then dilution series of analyte were applied to wells. After 30 min, wells were emptied and plates were washed. The detection antibody was then added to the wells, either polyclonal rabbit anti-ricin (85 ng/ml), biotinylated goat anti-ricin (20 ng/ml), or biotinylated mouse mAb (working concentration for each mAb determined by prior titration in this format). The final layer of the sandwich, HRP conjugate of goat anti-rabbit IgG (33 ng/ml, Zymed) or, for biotinylated antibodies, HRP-streptavidin (250 ng/ml, Zymed), was then applied. For all formats, after standard incubation and washes, assays were developed for 30 min with 100 µl/well tetramethylbenzidine (TMB) substrate (Enhanced K-Blue, Neogen, Lexington, KY, USA), followed by addition of 100 µl/well 0.3 N HCl. $A_{450}-A_{650}$ was determined using a microplate reader (M2, Molecular Devices, Sunnyvale, CA, USA), and standard curves were fitted to a logistic model using SoftMax Pro v4.7 (Molecular Devices) or SlideWrite v6 (Advanced Graphics, Encinitas, CA, USA). From the fitted curve, the IC_{50} – concentration of analyte resulting in half-maximal response – was computed.

Isotyping

Isotyping reagents were obtained from Zymed and Southern Biotechnology. Isotyping was generally performed on ricin-coated ELISA wells, following manufacturer's instructions, but with TMB substrate. Some samples were isotyped using goat anti-mouse Ig-coated wells.

Biotinylation of monoclonal antibodies

N-hydroxysuccinimidobiotin (Sigma), 0.4 ml of 1 mg/ml in dimethylsulfoxide, was added to 2 mg of IgG (0.5–2 mg/ml) in 2 ml of 0.2 M sodium borate buffer, pH 8.8. After 4 h, the reaction mixture was dialysed against PBS+0.01% NaN_3 , then stored at 4°C.

Protein and IgG determination

Protein concentration was determined using bicinchoninic acid (Smith et al., 1985), or spectrophotometrically for purified IgG (A_{280} , 1 mg/ml = 1.35).

Fluorescent staining of hybridomas

Log-phase hybridomas (1×10^6 cells/sample) were harvested by centrifugation. Before conducting labelling procedures, cells were pre-treated with 1% normal goat serum (NGS) for 15 min on ice. Cells were then treated for 15 min on ice with 100 µl of antibody or streptavidin reagent, as described below. Cells were washed three times with 1 ml of 1% NGS following each labelling step.

Direct staining of surface γ 1 chain

Recombinant phycoerythrin (rPE)-conjugated goat anti-mouse IgG1 (Invitrogen, Eugene, OR, USA) was used at a concentration of 50 μ g/ml in 1% NGS.

Indirect staining of surface μ and γ chains

Rabbit IgG anti-mouse μ , γ 1, and γ 2a chain reagents (Invitrogen) were biotinylated, as described above. Hybridomas were incubated with biotinylated antibody (100 μ l, 0.1–0.2 mg/ml), washed, and then stained with either fluorescein isothiocyanate (FITC) streptavidin or rPE-streptavidin (Invitrogen), 50 μ l at 40 μ g/ml. Cells were then washed three times with 1% NGS, resuspended in medium, and filtered as described below. In some experiments, cells were doubly labelled using two different biotinylated anti-isotype reagents and two streptavidin conjugates. In these experiments, cells were treated with iminobiotin to saturate biotin-binding sites of the first streptavidin conjugate, before proceeding with the second set of staining reactions.

Controls included cells stained with only secondary reagents and cells stained with an inappropriate anti-isotype reagents (e.g. γ 2a-secreting cells stained for γ 1). Before sorting, cell suspensions were filtered through CellTrics 30 μ m filters (GCAT, Fort Collins, CO, USA) and diluted in RPMI-1640, supplemented as for cell culture, but without serum.

Fluorescence-activated cell sorting (FACS)

Cells were analysed on a FACS Vantage SE flow cytometer equipped with a Coherent Enterprise II, water-cooled argon laser (Becton-Dickinson, San Jose, CA, USA). Cells were excited using 150 mW laser radiation at 488 nm. Fluorescence of the FITC and rPE fluorophores was monitored using 530/30 nm and 575/26 nm bandpass filters, respectively. Prior to sorting cells, the fluidics of the cytometer were flushed for 30 min with 5% H₂O₂ followed by 70% ethanol. After an overnight soak and morning flush with 70% ethanol, the lines were dried with sterile filtered air and then filled with sterile filtered sheath fluid (Hanks' balanced salt solution). Cells were sorted into polypropylene collection tubes using gating based on scatter and fluorescence intensity.

Sorted populations were collected into 12 $\times$ 75 mm polypropylene tubes containing culture medium with 15% fetal bovine serum, supplemented by 10% macrophage-conditioned medium (Sugasawara, Cahoon, & Karu, 1985). Depending on the experiment, cells were then distributed into 96-well plates at 1–10 cells/well. Antibody activity and isotype were assessed by ELISA after approximately 12 days of culture.

Results and discussion*Serum antibodies*

Both isolated chains of ricin are strong immunogens, and the results of screening mouse sera are summarised in Figure 1. The results show that the immune response was specific for the immunising antigen, with little cross-reactivity for the alternate chain. It should be noted that these quick screening assays were done by direct-

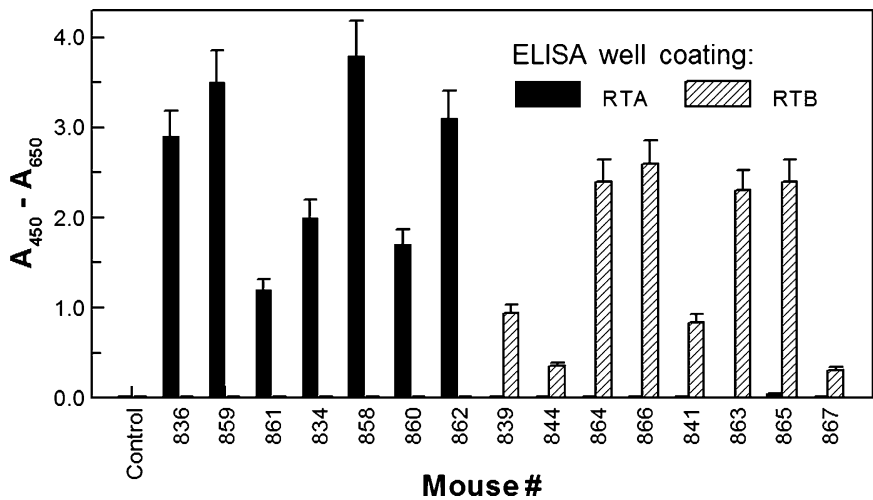


Figure 1. Response of mice to inoculation with isolated ricin A-chain or B-chain. A pool of normal mouse sera from unimmunised mice was used as control.

binding ELISA, with protein coated on plastic. The sandwich ELISA format and use of intact protein instead of isolated chains sometimes yielded different results (see below).

Monoclonal antibodies

Both groups of mice were utilised in cell fusion experiments to produce hybridomas. The initial screening results for some of these antibodies are summarised in Figure 2. It is apparent that most of the mAbs bind much better to the intact, dichain proteins than to the isolated chains. A possible explanation is that there is, essentially, random

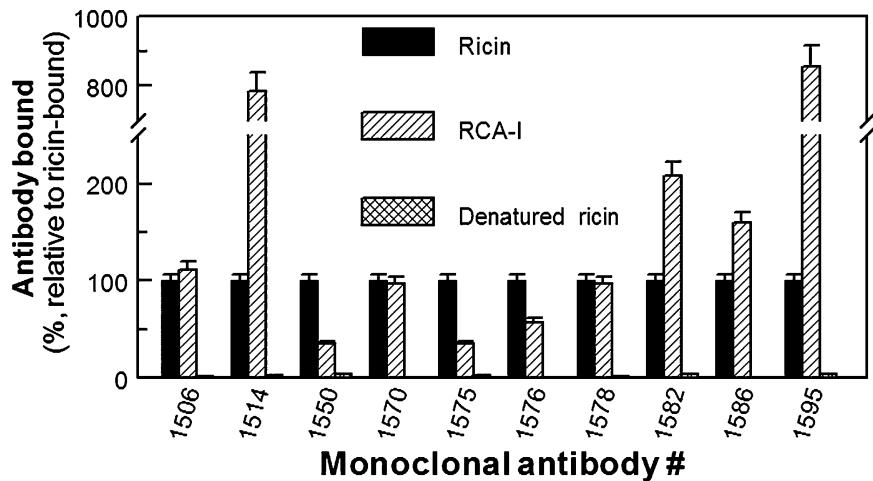


Figure 2. Binding of monoclonal antibodies to ELISA wells coated with ricin, heat-denatured ricin, or RCA-I. For each antibody, the binding to ricin was normalised to 100%.

Table 1. Specificity of mAbs for native ricin and apparent affinities.

mAb	IC ₅₀ ^a (ng/ml)	Binding to native relative to denatured ricin ^b
1409	0.25	22
1422	0.069	15
1428	0.16	15
1433	0.14	15
1435	0.076	13
1446	0.50	12
1457	0.17	7.0
1460	0.062	3.7
1471	0.15	8.8
1519	0.15	7.3
1522	0.55	7.2
1526	11	1.4

^aConcentration of ricin in solution resulting in 50% of maximal mAb binding to ricin-coated wells.

^b(Percentage (%) inhibition of binding by native ricin)/(Percentage (%) inhibition of binding by denatured ricin).

binding of the proteins to the ELISA wells, presumably by hydrophobic binding. For agglutinins bound predominantly via B-chain, more A-chain epitopes are accessible and displayed without potential distortion or steric hindrance owing to binding to plastic. The apparent distortion of proteins bound to plastic has been documented and discussed (Dierks, Butler, & Richerson, 1986).

Table 1 shows the apparent affinity of selected mAbs in ELISA, as determined by IC₅₀, as well as the relative binding of the mAbs to native versus denatured ricin. The mAbs developed typically bind ricin in the ng/ml (15 nM) range. Table 2 shows the isotype distribution of the anti-ricin mAbs (not including subclones). There is a striking difference in the isotype specificity of mAbs, depending on the eliciting immunogen. RTA elicited a predominantly IgG response; RTB, mostly an IgM response. It should be noted that the screening reagent (rabbit anti-mouse IgG, including both anti-heavy and light chain activity) gave a higher response to IgG than to IgM, so that the isotype distribution of the cloned hybridomas is probably skewed towards IgG mAbs. Because of this screening bias, the preponderant IgM response to RTB may be even more significant than implied by the results. We speculate that RTB has some mitogenic activity, stimulating non-memory IgM-bearing B-cell clones via lectin activity, whereas RTA acts as a typical protein antigen, eliciting an anamnestic IgG response after multiple inoculations. A number of the IgM-secreting hybridomas appeared to produce a second IgG isotype. Initial

Table 2. Isotype of monoclonal antibodies to *Ricinus* agglutinins.

Immunogen	n	Principal isotype (Percentage (%) of clones)	
		IgG	IgM
RTA	25	84	16
RTB	27	15	85
RTA/RCA-1	9	67	33

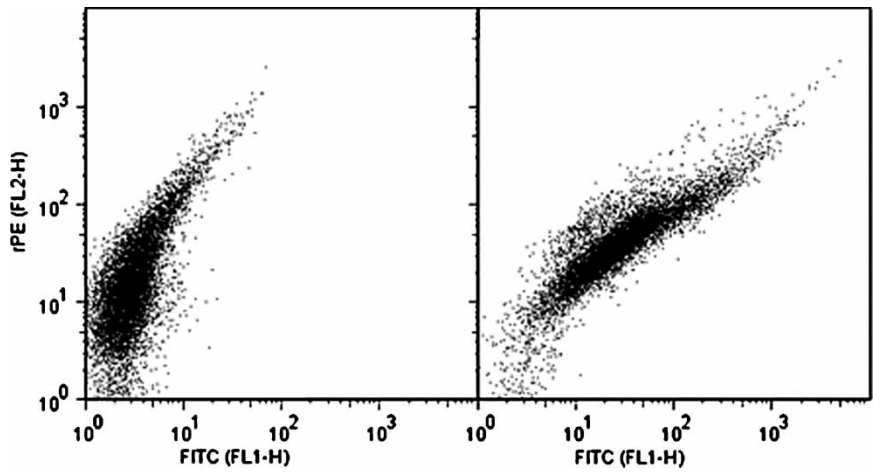


Figure 3. Plots of rPE fluorescence versus FITC fluorescence. On left, C1726 cells were labelled for γ 2a (biotinylated anti- γ 2a, then rPE-streptavidin) and for μ (biotinylated anti- μ , then FITC-streptavidin). On right, the cells were labelled in reverse order.

attempts to select single-isotype subclones were unsuccessful, and further analyses were conducted by FACS.

Figure 3 shows the FACS analysis of hybridoma C1726, initially characterised as secreting IgG2a, in addition to IgM. The dot plots indicate staining of cells for both isotypes, using biotinylated anti-isotype reagents and fluorescent streptavidin conjugates. From each staining experiment, 1000 of the brightest γ 2a-positive cells were collected, cultured, and analysed for isotypes expressed in the secreted antibody. In every culture analysed, isotyping revealed an apparently constant ratio of IgM to IgG production, using isotyping reagents from one supplier (Southern Biotechnology Associates). Analysis with isotyping reagents from a second supplier (Zymed), however, revealed that the original hybridoma as well as those selected by FACS were

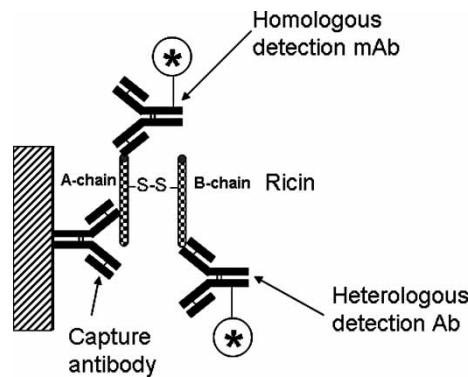


Figure 4. Schematic of principle of sandwich ELISA for ricin. Homologous assay can be conducted with a single mAb, unlabelled and labelled. Heterologous assays can employ a second, labelled mAb for detection or an antibody from a different species, labelled directly or visualised using a labelled goat anti-rabbit IgG, for example. In the schematic, capture via the A-chain is illustrated, but capture via the B-chain is also possible.

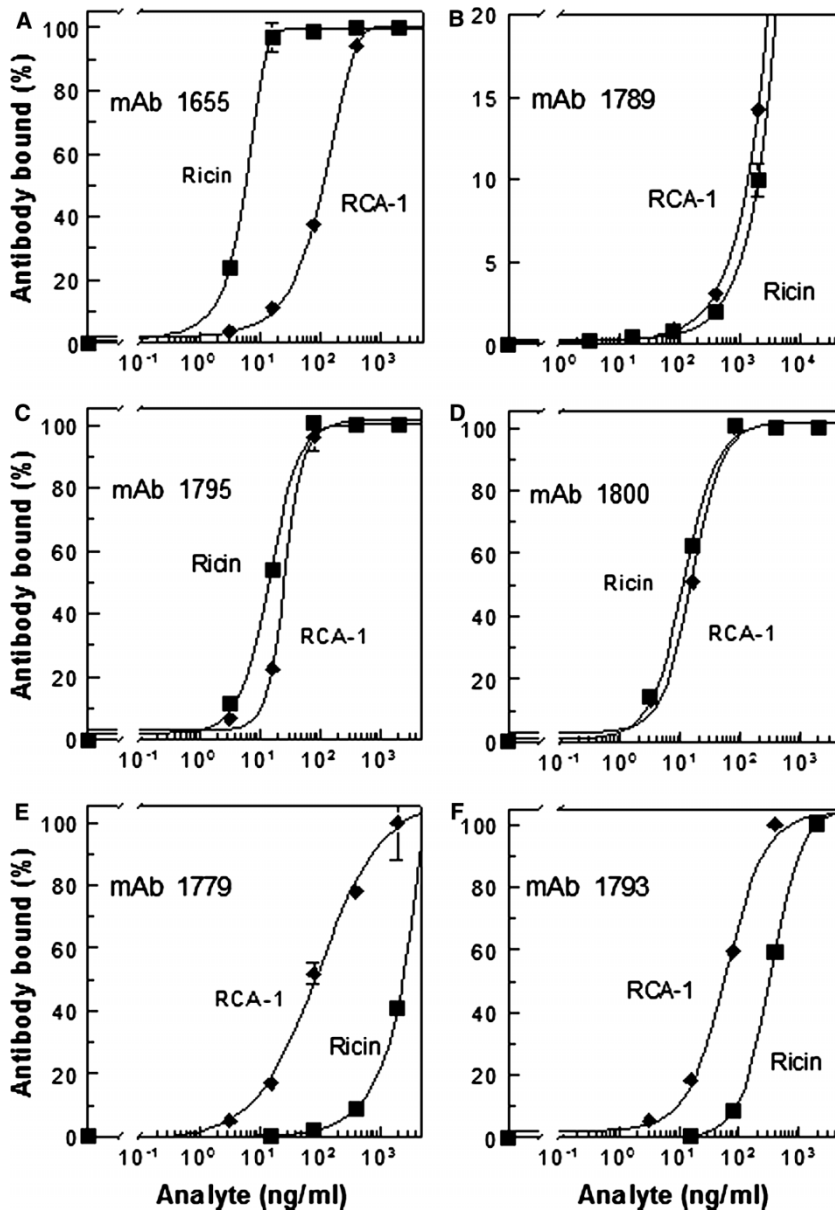


Figure 5. Heterologous sandwich ELISA with capture mAbs immobilised via goat anti-mouse IgG and detection with rabbit polyclonal anti-ricin and HRP-goat anti-rabbit IgG. Different patterns of specificity and affinity are observed. MAb 1655 elicited with RTA; the other mAbs, RTA, followed by booster with RCA-1. (A) 25-fold greater apparent affinity for ricin. (B) Apparent affinities of this IgM mAb for ricin and RCA-1 are low, but within two-fold of each other. (C) Five-fold greater apparent affinity for ricin. (D) Apparent affinities for ricin and RCA-1 within two-fold of each other. (E) 500-fold greater apparent affinity for RCA-1. (F) Six-fold greater apparent affinity for RCA-1.

all negative for $\gamma 2a$. Antibody 1726 was also analysed by affinity chromatography on Protein G-agarose. The antibody was found in the flow-through fraction only, with essentially no activity retained and eluted at low pH. We conclude that mAb 1726 is an IgM that expresses an epitope recognised by one of the $\gamma 2a$ -specific reagents, but not the second. We have identified two other IgM antibodies that cross-react with a γ -specific isotyping reagent from one of the two suppliers; in one case $\gamma 1$, and, in the other, $\gamma 2b$.

Use of the antibodies in ELISA

For a variety of reasons, sandwich assays are often preferable in complex matrices such as food, for example, to minimise matrix interference with the detection phase of the analysis. We therefore explored the use of these mAbs in sandwich ELISA, as depicted in Figure 4. Six typical patterns of specificity achieved using heterologous sandwich ELISA are shown in Figure 5. Because the detecting antibody (a commercial polyclonal Ab) binds to both principal analytes, the different results depend largely on the specificity and affinity of the capture mAb in each assay. It should be noted that panel B illustrates the results obtained with a relatively low affinity IgM antibody. This response was seen especially with monoclonals derived from immunisations using RTB. It occurred to a lesser extent in other experiments.

Figure 6 shows the results of a heterologous sandwich ELISA, employing mAb 1807 obtained in this study, using the polyclonal detection system. This combination produces an assay highly selective for RCA-1. Figure 7 shows the use of one sandwich format in a skim milk matrix. For this assay, there was no significant matrix effect, demonstrated by the fact that all matrix analyses fell within the 95% prediction interval of the standards in buffer. Because the two agglutinins occur in approximately equal concentration in extracted castor mash (crude ricin-rich preparations after the high quality castor oil is removed), a screening assay that detects both ricin and RCA-1 (e.g. Figure 5D) may provide enhanced sensitivity compared to a ricin-specific assay. Further studies will determine the appropriate sandwich format appropriate for various complex food matrices of interest.

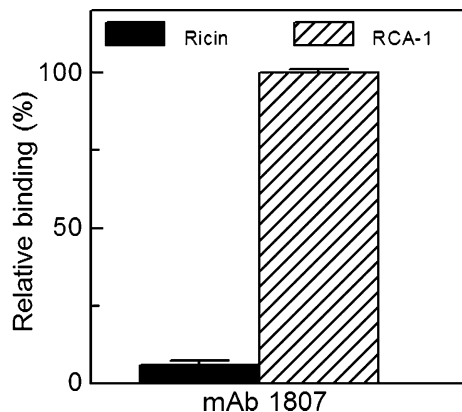


Figure 6. Heterologous sandwich ELISA with goat anti-mouse IgG/mAb 1807 and detection with rabbit polyclonal anti-ricin and HRP-goat anti-rabbit IgG.

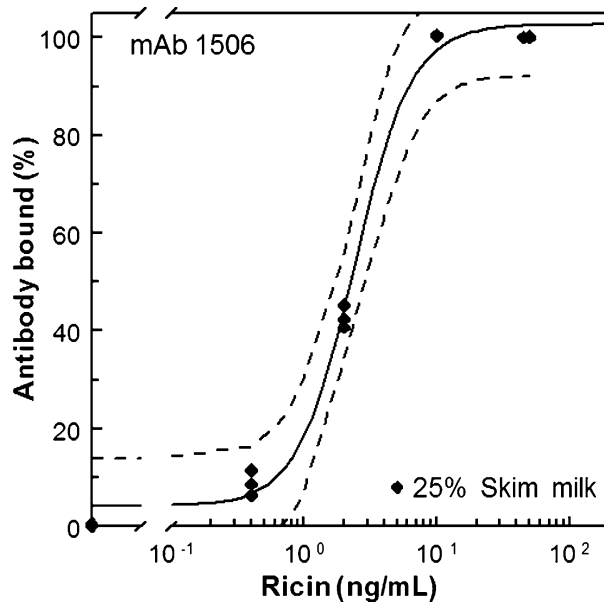


Figure 7. Analysis of ricin in 25% skim milk matrix by heterologous sandwich ELISA. Purified mAb 1506 (IgG₁κ) was used for capture, and biotinylated goat anti-ricin followed by HRP-streptavidin were used for detection. The standard curve obtained in buffer is shown, along with the 95% prediction interval.

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