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Shorter development of immunoassay for drugs: application of the novel RIMMS technique enables rapid production of monoclonal antibodies to ranitidine

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Abstract

High affinity, specific murine monoclonal antibodies have been produced for ranitidine using the novel RIMMS (repetitive immunizations, multiple sites) technique. We demonstrate that this technique can be employed to produce high affinity monoclonal antibodies to drug haptens in ~ 1 month; whereas, conventional techniques typically require 3–9 months. Polyclonal antiserum development typically requires at least 6 months. Consequently, RIMMS has a clear impact allowing reagent antibodies to be available earlier in the drug development process. Isotyping studies demonstrated that the developed antibodies are either IgG_1 or IgG_2b immunoglobulins which confirms that the technique produces class-switched, affinity matured reagent antibodies. The most promising monoclonal antibody for quantitative applications afforded similar sensitivity, by competitive ELISA, to the established sheep polyclonal anti-ranitidine sera. The calibration range, estimated as the limits between the asymptotic regions of calibration graphs, is 0.5–41.2 ng ranitidine per well. Specificity studies indicated that the monoclonal antibody afforded superior selectivity, yielding only 4.1% cross-reactivity with the ranitidine sulphoxide metabolite; the corresponding value for the antiserum was 8.6%. Both reagents had similar cross-reactivities with the *N*-oxide metabolite.

Keywords: RIMMS technique; Ranitidine; Isotype

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1. Introduction

Immunoassay is employed widely in pharmaceutical development for high-throughput and high-sensitivity determination of drug levels in biological samples generated during pre-clinical and clinical pharmacokinetic studies. However, monoclonal antibodies (MAbs) are not generally used for drug haptens because affinities are often low and limit assay sensitivity. Furthermore, production times are excessive when compared to the generation of polyclonal antisera [1,2]. Nonetheless, monoclonal antibodies are desirable because purification of immunogens need not be as rigorous as that required for the generation of antisera, and they can be selected on the basis of desired specificity. This latter feature is particularly important for the bioanalysis of drugs because analytical methods are required that are free from interferences caused by drug metabolites.

We have recently reported a novel technique that capitalizes on primary T cell dependent B cell responses for the rapid production of murine monoclonal antibodies [3]. This technique employs a novel 8–13 day immunization regimen followed by fusion of murine lymphocytes to a myeloma fusion partner expressing the Bcl-2 proto-oncogene product. The monoclonal antibody development program, including immunizations, fusions, screening and cloning, generally takes ~ 30 days to complete. Immunizations and fusions are completed within 9–14 days after commencing immunizations; screening is typically performed between days 17–22, with cloning and confirmation of Mab specificity generally completed during days 17–30. The corresponding time frame for developing MAbs by means of established techniques is typically 3–9 months. Consequently, RIMMS has a clear impact allowing antibodies to be available earlier during the drug development process.

In this paper, we report the first application of the innovative and rapid production technique designated 'RIMMS' (repetitive immunizations, multiple sites), for the production of monoclonal antibodies suitable for the immunoassay of a drug hapten. Ranitidine was selected as the drug hapten for our investigation because there was an established radioimmunoassay employing a polyclonal antiserum that could be used for comparison [4]. Our aim in this study was to determine whether the RIMMS technique could generate monoclonal antibodies suitable for the determination of drug haptens. It was not our intention to develop a bioanalytical method because a robust and reliable method already exists for the determination of ranitidine in biological fluids.

Ranitidine, a H2 blocker that is the active component in Zantac, is the most successful medication prescribed for the treatment of duodenal ulcers. Ranitidine is an important component in ranitidine bismuth citrate (Pylorid/Tritec) that has proved efficacious in treating ulcers and also eradicating *H. Pylori*, the bacterium implicated as a causative agent in some gastro–intestinal diseases. More recently, ranitidine has become available as an over-the-counter medication for the treatment of acid indigestion.

In this report, we describe synthetic routes for the synthesis of ranitidine immunogens, the RIMMS immunization regimen in mice, and the fusion and screening strategies for monoclonal antibody production. Several monoclonal antibodies were then compared to the anti-ranitidine serum that is used in the established competitive radioimmunoassay. These studies demonstrate that the most promising monoclonal antibody is at least equivalent to the polyclonal antiserum for the determination of ranitidine.

2. Material and methods

2.1. Materials

Buffer salts (analytical reagent grade) were purchased from either Aldrich or J.T. Baker; organic solvents were obtained from EM Science. Ranitidine as the hydrochloride salt, the hapten, and the ranitidine analogues were supplied by Glaxo Wellcome Research and Development. Bovine serum albumin (BSA) and bovine thyroglobulin (BTG) were from Sigma (St. Louis, MO); Keyhole Limpet haemocyanin (KLH), ovalbumin (OVA) and dialysis cassettes (Slide-A-Lyzer) were purchased from Pierce. RIBI's adjuvant and

polyethylene glycol 1450, were purchased from RIBI and ATCC, respectively. Freund's complete adjuvant was obtained from GibcoBRL. The alkaline phosphatase (ALP)-labelled γ-chain specific secondary antibody, and the isotype and subclass-specific antibodies were obtained from FisherBiotech (Pittsburgh, PA). EIA/RIA, 96 and 24 well tissue culture plates and all cell culture vessels were purchased from Costar.

2.2. Preparation of cell culture media and reagent solutions

All cell culture media were prepared aseptically and sterile filtered before use.

Hybridoma medium (per 100 ml): 50 ml EXCELL 610 media (JRH), 10 ml 10% supplemented ORIGEN cloning factor (IGEN, Fisher), 1.0 ml 200 mM L-glutamine (Gibco), 50 μl 0.1 M β-mercaptoethanol, 1.0 ml 10 000 U ml^{-1} penicillin-10 000 U ml^{-1} streptomycin stock (Gibco), 10 ml fetal calf serum, 1.0 ml 10 mM non-essential amino acids (Gibco), 27 ml RPMI 1640 media.

HAT Selection medium (2x stock per 100 ml): 96 ml Hybridoma media, 4 ml 50X HAT (5 mM hypoxanthine, 0.02 mM aminopterin and 0.8 mM thymidine, Sigma).

HT medium (1x stock per 100 ml): 100 ml Hybridoma media, 1 ml 100X HT (10 mM hypoxanthine, 1.6 mM thymidine, Sigma).

ELISA coating buffer: 0.1 M carbonate buffer, pH 9.6.

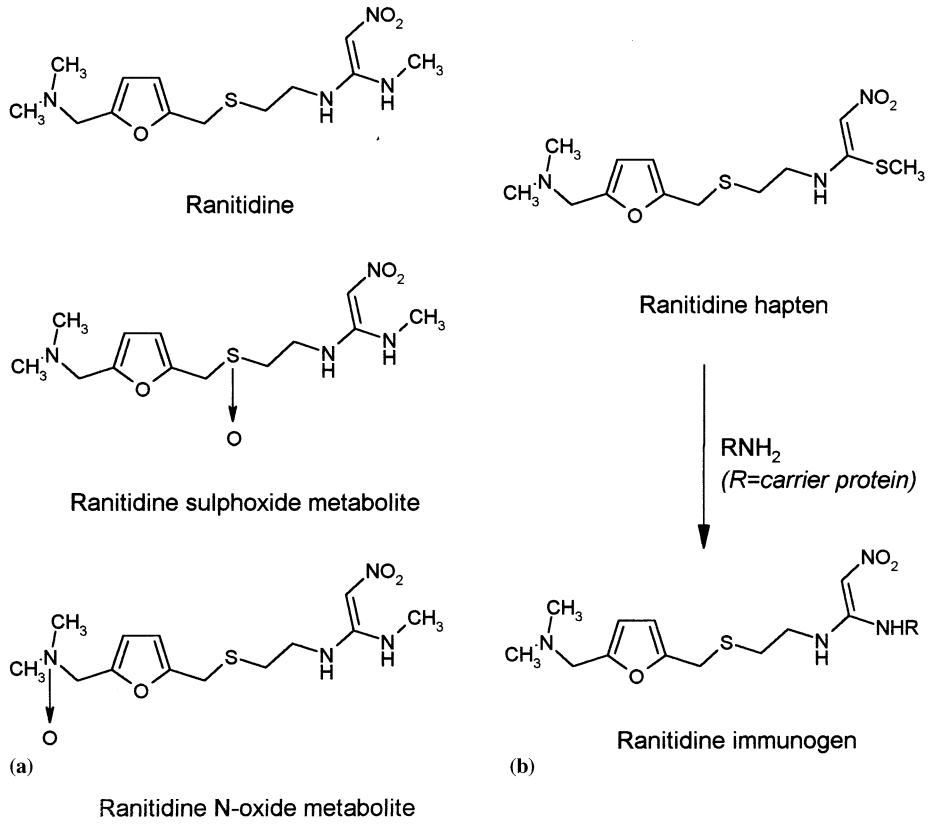


Fig. 1. Structures of (a) ranitidine and its N-oxide and sulphoxide metabolites; and (b) the synthetic route for the ranitidine immunogens.

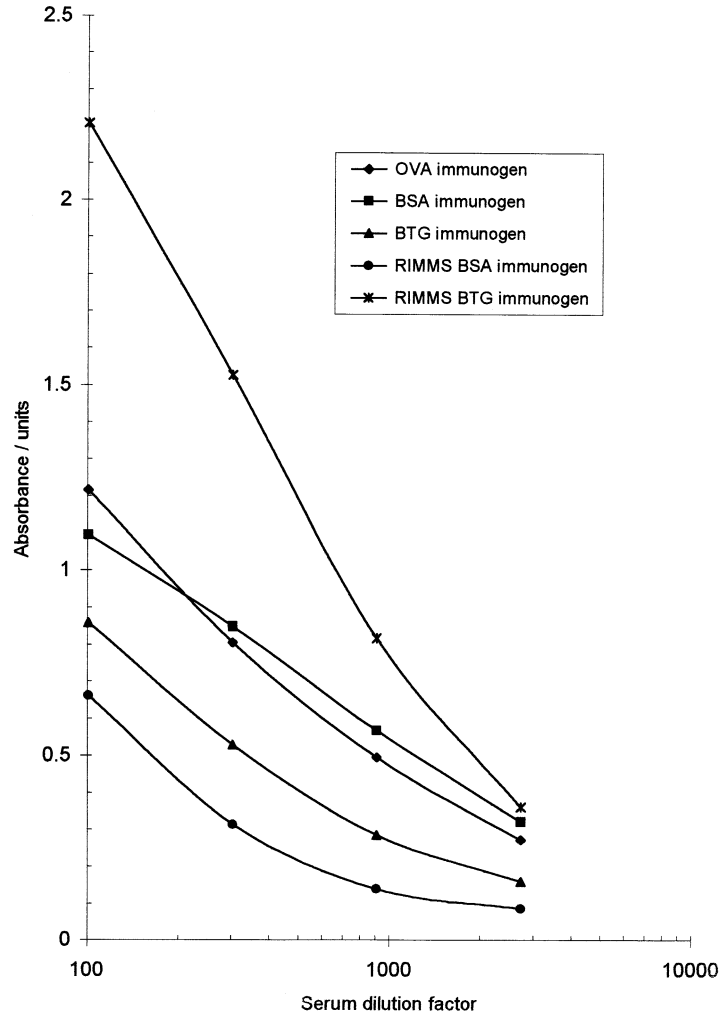


Fig. 2. Dilution curves for anti-ranitidine sera taken from mice given immunogens by conventional immunizations and RIMMS.

ELISA diluent: 0.1 M phosphate buffered saline (Gibco)

ELISA blocking buffer (per 100 ml): 5 ml of either normal goat or horse serum (for use with murine monoclonal or sheep polyclonal antibodies, respectively), 95 ml tricine buffered saline.

ELISA wash solution (per 100 ml): 50 μl Tween-20, 100 ml Tris buffered saline.

ELISA alkaline phosphatase substrate buffer: 48 ml Diethylamine (Sigma), 400 ml deionized water, 50 mg $MgCl_2 \cdot 6H_2O$, adjusted to pH 9.8 with 1M HCl, bring volume to 500 ml with deionized water.

ELISA alkaline phosphatase substrate: One Substrate-104 tablet (5 mg *p*-nitrophenyl phosphate, Sigma) per 5 ml substrate buffer.

Ranitidine and analogue solutions: Primary stock solutions containing 1 mg ml^{-1} of ranitidine base were prepared monthly in deionized water and stored at $\sim 4°C$. Working cali- bration standards were prepared on the day of analysis by diluting the primary stock solution in either ELISA diluent or drug-free human serum. Solutions of compounds used for cross-reactivity studies were prepared similarly.

2.3. Apparatus

Cell culture was performed using standard equipment. ELISA plates were washed in a Skatron A/S Microwash II 96-well plate washer and read at 405 nm in a Molecular Devices Thermomax plate reader.

2.4. Synthesis of the ranitidine–protein conjugates

Conjugates could not be prepared with ranitidine directly because the compound does not possess suitable functional groups for coupling to carrier proteins. Therefore, an *S*-methyl ranitidine analogue (AH21031XX; Fig. 1) was synthesized in which an *S*-methyl group replaced the methylamine group in the parent compound. Conjugation to BSA, BTG and KLH carrier proteins was performed according to the method described for BSA by Jenner et al. [4] The conjugates with BSA and BTG were prepared for immunization, and the KLH conjugate was used for coating ELISA plates.

The ranitidine -BSA and -BTG immunogens (Fig. 1) were produced by stirring 60.6 mg (0.18 mmoles) of hapten and 40 mg of protein (615 nmol BSA, 61 nmol BTG) in 5 ml deionized water for 4 days at an ambient temperature. Whereupon, the protein solutions were centrifuged and the supernatants dialysed, in Slide-A-Lyzer

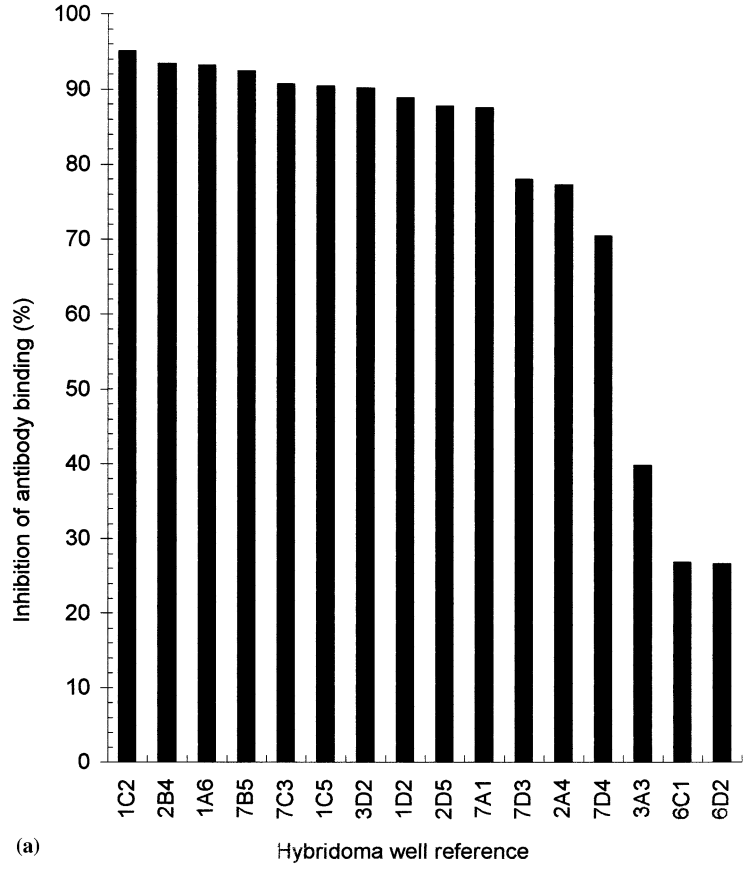
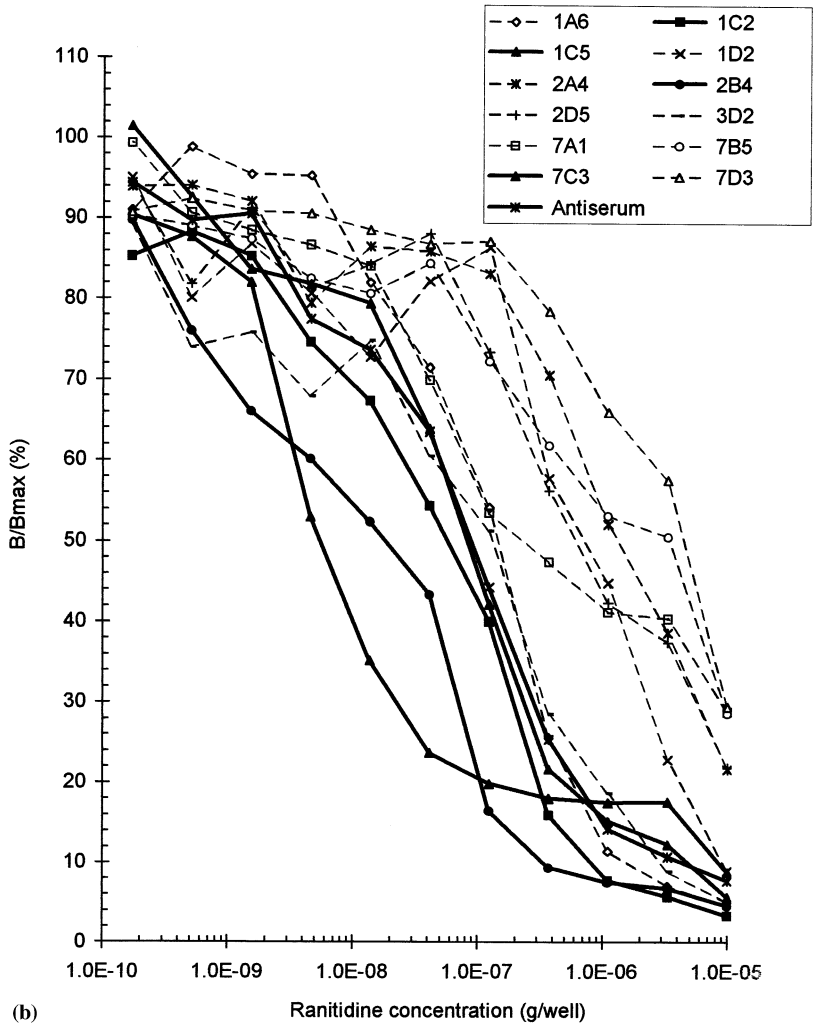


Fig. 3. Histograms (a) showing the inhibition of antibody binding in the presence of ranitidine; and (b) mean displacement curves, from the ELISAs used to screen for ranitidine antibodies in the hybridoma supernatants; data for hybridomas taken for cloning are presented as solid curves. (b) presents mean data from assays run on the supernatants (described by plate reference) collected on three occasions over 7 days; data are also included for pooled antisera collected from the mice at euthanasia.



(b)

Fig. 3. (*Continued*)

(Pierce) cassettes, for 72 h against several changes of deionized water. The purified conjugate solutions were further purified by gel filtration chromatography (Sephadex G25 packed in a 40×1.0 cm i.d. column; eluent, 0.1 M sodium chloride; flow rate 1 ml min^{-1}). The conjugates, which eluted from the column in the void volume, were collected, dialyzed against deionized water, and freeze-dried to yield ~ 35 mg of each protein. Both conjugates were pale yellow in color; the BSA immunogen was soluble, while the BTG conjugate was only sparingly soluble. Both immunogens were stored as solids at 4°C.

The KLH-conjugate was synthesized using a similar method except that 15 mg of hapten were dissolved in 200 μl of DMSO and then added to a vial containing 20 mg of Imject KLH (Pierce) reconstituted in 2 ml of deionized water. The conjugate was purified by dialysis against 0.1 M NaOH, aliquoted and stored at -20°C.

The incorporation of hapten into the BSA and KLH conjugates, determined using UV spectroscopy at 320 nm (nitro-benzene moiety), was 4.3 and ~ 117 mol per mole of protein, for the BSA and KLH conjugates, respectively. Incorporation could not be determined for the BTG

immunogen because of poor solubility; however, the UV spectrum confirmed successful conjugation.

2.5. Immunization of mice

In a preliminary study, two 8-week old male SJL mice were immunized with either ranitidine-BSA or ranitidine-BGT immunogens, (one mouse per conjugate). Each animal received immunogen on six occasions over a 13 day immunization regimen. The amounts inoculated were 30, 15, 6, 3, 3, and 3 μg on days 1, 3, 6, 8, 10 and 13, respectively. On each occasion, the immunogen was dissolved in 400 μl of sterile phosphate buffered saline (PBS) and divided for delivery with two adjuvants—one half was emulsified with 200 μl Freund's complete adjuvant, and the other half was mixed with 200 μl RIBI's adjuvant. Each adjuvant was injected subcutaneously to bilateral sites (calf, groin and axillary) adjacent to draining lymph nodes, using a modification of the proce-

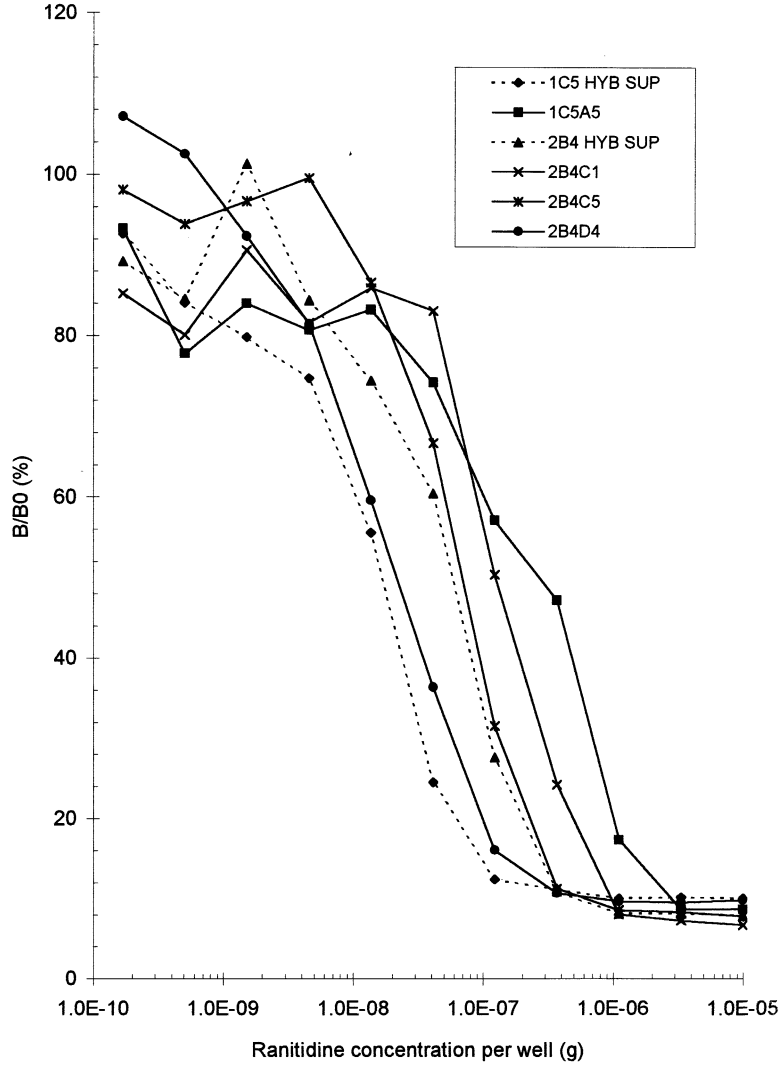


Fig. 4. ELISA displacement curves for ranitidine using cell culture supernatants from the most promising hybridomas before and after cloning (B/B_0 (%), binding expressed as a percentage of maximum binding).

Table 1
Cross-reactivity data (%) for ranitidine monoclonal antibodies and the established sheep anti-ranitidine serum

Compound	Monoclonal antibody				Sheep polyclonal[b]
	1C5A5	2B4C1	2B4C5	2B4D4	
N-oxide metabolite	<1.0[a]	0.7	0.3	0.2	0.3 (0.1)
S-oxide metabolite	70.7	5.8	5.1	4.1	8.6 (10.0)

[a] Estimated value.
[b] Values in parentheses are cross-reactivity data obtained by RIA.

dure described by Caterson et al. [5]. The immunogen in RIBI's adjuvant was also delivered to two sites in the nape of the neck. Mice were anesthetized with isoflourane for all immunizations.

Subsequently, two additional mice were immunized with the ranitidine-BTG immunogen using the same immunization regimen.

2.6. Fusion protocol

On day 14, a single cell suspension was prepared aseptically in RPMI-1640 medium from popliteal, superficial and deep inguinal, axillary, and brachial lymph nodes bilaterally harvested from both animals. The suspension was prepared in a Petri dish by teasing the nodes apart with curved forceps; the media containing the cells was aspirated, excluding large particulate matter, and transferred to a sterile centrifuge tube making up to 15 ml final volume with serum-free RPMI-1640. The cells were then centrifuged ($400 \times g$) and washed with serum-free RPMI-1640. This step was repeated; whereupon the suspension was added to similarly washed P3XBcl-2-13 cells at a ratio of $\sim$2.5:1. The mixed cell suspension was centrifuged and the supernatant was removed completely. The combined lymphocytes and P3XBcl-2-13 cells were fused using a modification of the method described by Kilpatrick et al. [6]. To summarise, 0.5–1.0 ml of 34% (m/v) polyethylene glycol 1450 (PEG) diluted in serum-free RPMI-1640 was added to the mixed cell pellet in a 50 ml conical tube over 1 min. The PEG-cell suspension was gently mixed for 1 min., whereupon 13 ml of RPMI-1640 was added dropwise to the tube over 8 min. The temperature was maintained at 37°C throughout. The cell suspension was then centrifuged for 7 min; afterwards the pelleted cells were resuspended gently in 48 ml of hybridoma medium. The fusion suspension was distributed as 1 ml aliquots into 24 well plates. After 24 h in culture, 1 ml 2*x* HAT selection medium was added to each well. Media was changed to 1*x* HAT medium also containing 200 μg ml^{-1} G418 after an additional 72 h in culture. G418 is a selection marker for the P3XBcl-2-13 cells containing the transferred Bcl2 gene. G418 should not be used with parent P3X cells.

2.7. Cloning of hybridomas by cell sorting

Fluorecence-activated cell sorting was employed to distribute single cells from promising hybridoma pools to 96-well plates. This step is performed as soon as antigen relevant antibodies are detected in hybridoma supernatants; typically 5–10 days after fusion. Immediately prior to sorting, the cells were harvested and suspended in fresh HAT medium at room temperature. The cells were introduced into the sample stream of a Becton Dickinson FACStar$^{Plus™}$ flow cytometer equipped with an argon ion laser in the primary light path (60 mW at 488 nm, Ion Laser Technology, Salt Lake City, UT). A nozzle diameter of 100 μm was employed and droplets were generated by a macroSORT® (Becton Dickinson) transducer operating at 17 000 Hz. System fluid pressure was maintained at 7 psi and events were detected on a forward light scatter (FSC) trigger. The signals generated for FSC, side scatter (SSC) and FSC pulse width (FSCW) were monitored as linearly amplified quantities and the signal for autofluorescence at 535 nM was processed as a

log-amplified value. The autofluorescence signals were derived via a 530/30 band-pass filter (BP) following transit of a 560 short-pass dichroic filter (DF). A rectangular sort region was defined by producing Cartesian plots displaying FSC versus SSC values to delineate the predominant cell population. To minimize the sorting of coincident groups of cells, a second population was defined by a Cartesian plot of autofluorescence at 535 nM versus FSCW. Care was taken to identify and gate on the compact FSCW population representing single cells. Using a robotic positioner and dedicated software the instrument was set to deliver single cells in three-drop packets to each well of a 96-well plate containing 200 μl HT medium/well. The cytometer was routinely checked for alignment and target fluorescence values were set and checked using a glutaraldehyde-fixed chicken red blood cell standard immediately before and after sample batches were run. In addition, the

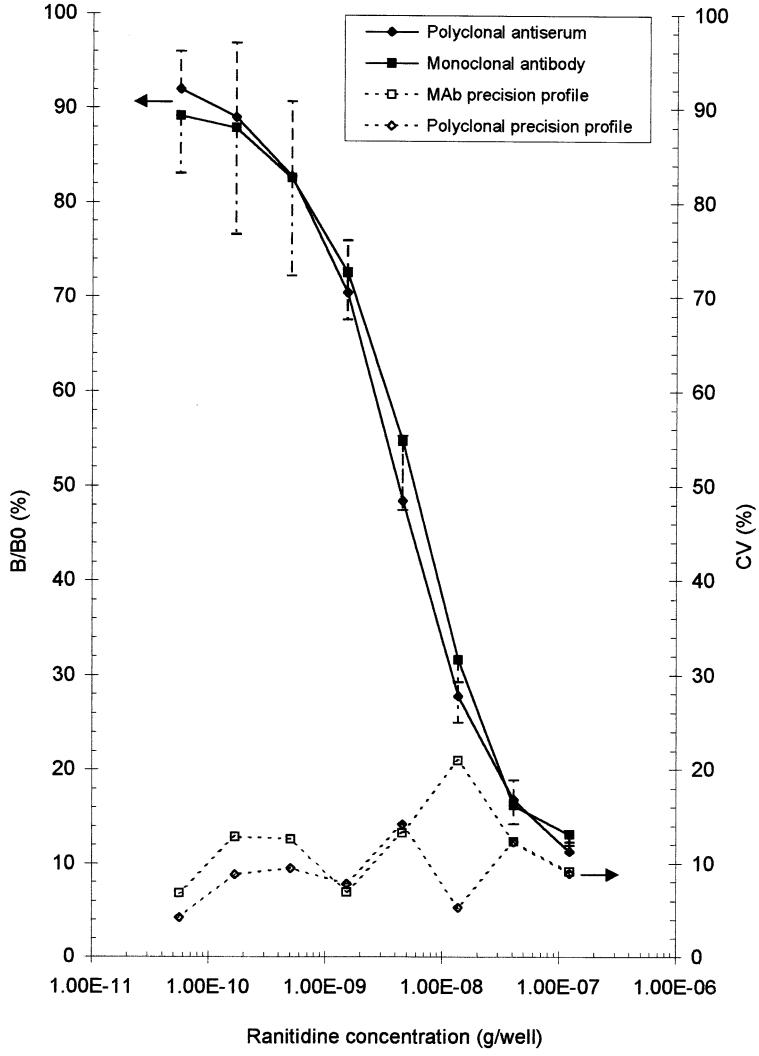


Fig. 5. Mean ± SD calibration graphs and precision profiles ($n = 8$) for the optimized ELISA of ranitidine using the RIMMS murine MAb (2B4D4) and the reference sheep anti-ranitidine serum. Antibody concentrations were matched to give similar absorbance readings for the zero standard (B_0) wells.

sorting efficiency of the instrument was checked using a mixture of labeled microspheres in the performance of a 'test sort' using a one-drop packet.

2.8. Enzyme-linked immunosorbent assay (ELISA)

By incubating, 96 well EIA/RIA plates were coated with between ~50 ng and 1 μg per well of ranitidine-KLH conjugate dissolved in 0.1 ml of coating buffer. The conjugate solution was removed and the wells were blocked by incubating with 0.15 ml of blocking buffer. The wells were then washed with blocking buffer and 50 μl of the supernatants from growth positive wells were added and the plate reincubated. After washing three times with washing buffer, an affinity purified alkaline phosphatase-labelled goat anti-mouse IgG γ-chain specific secondary antibody was added, at the manufacturers recommended titre. Each incubation step was performed for either ~60 min at 37°C or overnight at 4°C.

Wells were washed six times and the color development was performed at an ambient temperature using a Sigma 105 alkaline phosphatase substrate according to the manufacturer's recommendation. Absorbances for each well were determined at 405 nm.

This method was also used for competitive ranitidine displacement ELISA and to isotype the ranitidine-reactive monoclonal antibodies. For competitive ELISA, 0.01 ml per well of ranitidine standard or spiked serum was added prior to the addition of ranitidine monoclonal antibody. Isotyping was performed using ALP-labeled, isotype and subclass-specific secondary antibodies rather then the IgG γ-chain specific reagent described above.

Cross-reactivity curves were constructed using the competitive ELISA with solutions of a test compound substituted for the ranitidine standards.

The established sheep anti-ranitidine serum was evaluated using the ELISA method described above with an alkaline phosphatase-labelled Donkey anti-sheep IgG for the secondary antibody, and normal horse serum was employed for blocking.

3. Results and discussion

3.1. The RIMMS technique for MAb production

The RIMMS technique provides bioanalysts with an opportunity to produce and isolate affinity matured, class switched (IgG) monoclonal-antibody-producing cell lines typically within 30 days of the primary immunization. This would require 3–6 months using conventional techniques. We have demonstrated previously that B lymphocytes can be driven to undergo rapid affinity maturation within secondary lymphatic tissues using an abbreviated immunization regimen [3]. Our findings are supported by earlier reports which describe T cell dependent B cell responses following an antigenic challenge. The novelty of RIMMS arises from several adaptations of established procedures which are introduced during immunization and somatic fusion.

3.2. Immunization strategy

A multiple site, dual adjuvant immunization regimen is employed. Immunogen is emulsified separately with Freund's and RIBI's adjuvants for subcutaneous delivery to several sites adjacent to peripheral lymph nodes. This latter step is important because these immunization sites target drainage to regional nodes in order to maximize immunorecognition. Regional nodes are known to be involved in primary and secondary T cell dependent responses which makes them a valuable source of immunoresponsive cells [7–9]. The immunization regimen involves doses (μg) of immunogen delivered at 2–3 day intervals over 13 days. Adjuvants promote the recruitment of T helper cells that produce cytokines, which influence the isotype and subclass of antibodies produced during antigenic challenge. The dual adjuvant strategy enhances cytokine production, and diversifies and encourages the early expansion of the peripheral lymph node B cells that give rise to antigen-specific antibodies.

Affinity maturation and selection of antibodies occurs within the newly formed germinal centers of secondary lymphatic tissue 5–14 days after immunization. By having frequent immunizations

adjacent to lymph nodes, we were able to maximize the delivery of antigens to the target tissue containing antibody producing B cells.

3.3. Polyclonal titres

Mice were immunized with either the ranitidine-BTG or ranitidine-BSA immunogens in a preliminary study using the RIMMS strategy, and also the conventional regimen for comparison. In addition, ranitidine-OVA immunogen was also administered by the latter regimen. Test bleeds collected from all mice were titered to assess antisera development. All immunized mice responded and mean antisera dilution curves are presented in Fig. 2. Interestingly, within the limitations of the data, the magnitudes of the antibody responses were not markedly different; although the best responses were obtained in the mice receiving the BTG immunogen by the RIMMS regimen. Consequently, this immunogen was used for further RIMMS immunizations. No further work was performed with the mice immunized by the conventional regimen.

RIMMS has now been employed successfully for over 20 monoclonal antibody generation programs and where data are available, we generally see a good polyclonal response with RIMMS. However, there have been instances where there was no detectable polyclonal response following RIMMS. Nonetheless, high affinity monoclonal antibodies were produced. This indicates that by day 13, when lymph nodes are harvested, polyclonal titer is not always reflective of underlying B cell maturation and the selection process that is evolving in lymph nodes.

3.4. Fusions

Somatic fusions are performed, before immunodominancy is established, between lymphocytes and a P3XBcl-2-13 cell line (fusion partner) that over-expresses Bcl-2. Immunodominancy occurs during primary T cell-dependent B cell responses due to selective processes that occur within germinal centers in secondary lymphatic tissue. Selection involves signaling and co-stimulatory events within germinal centers, which occur as a result of the expression of antigen-specific affinity-matured surface immunoglobulin receptors. Germinal centers also play an important role in the deletion of immunoreactive self antibodies [7,10]. The Bcl-2 gene product plays an important role in vivo in regulating the expansion and selection of B cells within germinal centers [11,12]. During somatic fusion, a fusion partner that expresses Bcl-2 can usefully rescue weak hybridoma cells from cell death. This leads to a greater yield of viable hybridomas, thus increasing the repertoire of antibody production. Indeed, Ray and Diamond [13] demonstrated a 2- to 5-fold increase in hybridoma outgrowth of splenocytes fused with NSO cells stably transfected with Bcl-2 ($NSO^{bcl\text{-}2}$). We have also found that hybridomas are more robust using our P3XBcl-2-13 fusion partner, which also aids in increasing cell survival during subsequent cloning of hybridomas.

Lymph nodes were harvested and pooled from the two SJL mice that received the ranitidine-BTG immunogen by the RIMMS immunization regimen. A single-cell suspension was prepared, which yielded $\sim 4.8 \times 10^7$ cells with 80% viability as determined by typan blue staining. Lymphocytes were fused with $\sim 2 \times 10^7$ P3XBcl-2-13 cells representing a 2.5:1 ratio. The resulting hybridomas were plated into seven 24-well plates.

3.5. Screening hybridoma supernatants

Hybridoma supernatants were screened for ranitidine antibodies by employing an IgG (γ-chain) specific ELISA, and 16 wells yielded positive results with absorbance values three-times greater than the background. An IgG (γ-chain) specific ELISA was employed to exclude typically low affinity IgM antibodies. The positive supernatants were then evaluated in a competitive ELISA to confirm specificity for ranitidine. Fig. 3 shows that free ranitidine could inhibit antibody binding to the ELISA plates by at least 80% for 12 out of the 16 positive supernatants. The supernatants displaying poor inhibition are thought likely to contain low affinity IgG antibodies or antibodies that recognize the bridge region in the ranitidine-KLH conjugate coated onto the ELISA plate.

Ranitidine displacement (calibration) curves were then constructed for the 12 most promising supernatants (Fig. 3(b)), and as expected, absorbance decreased with increasing ranitidine concentration. Four hydridomas producing the supernatants that afforded the steepest and most sensitive curves were selected for cloning by either limiting dilution or flow cytometry. These curves had at least comparable sensitivity (determined by ED20, ED50 and ED80) to those obtained for the pooled polyclonal antiserum harvested from the mice at euthanasia. Isotyping of supernatants from the hybridomas selected for cloning revealed that three produced IgG_1 antibodies while the fourth produced IgG_2b. These data, and those obtained by the γ-chain (IgG) specific screening ELISA, confirm that the RIMMS technique gives rise to class-switched, affinity mature antibodies.

3.6. Cloning

Cell viability after cloning, determined by the number of wells showing cell growth, was 45 and 57% for limiting dilution (performed at a concentration of 0.2–0.3 cells/well) and flow cytometry, respectively. These data suggest that the techniques afford similar cell viability; however, flow cytometry is considered advantageous because it is markedly less labor-intensive.

Fig. 4 presents pre-cloning graphs and calibration graphs for supernatants collected after cloning from the most promising hybridoma supernatants. These graphs confirm successful isolation of the cell lines that produced the desirable antibodies identified during initial screening.

3.7. Specificity

Metabolism studies in humans have shown that the majority (68%) of a 100 mg intravenous dose of ranitidine is eliminated unchanged in urine [4]. Other drug-related material is mainly comprised of *N*-oxide (5.1%), *S*-oxide (1.7%) and *N*-desmethyl (2.4%) metabolites.

The potential specificity of an ELISA employing the ranitidine MAbs was assessed by constructing cross-reactivity curves for the *N*-oxide and *S*-oxide ranitidine metabolites; pure desmethyl ranitidine was unavailable. The data presented in Table 1 indicates that MAb 1C5A5 may be unsuitable for bioanalytical applications because of high cross-reactivity with the *S*-oxide metabolite. Monoclonal antibody 2B4D4 is the most selective and appears to be superior to the established sheep anti-ranitidine serum because of lower cross-reactivity with the *S*-oxide metabolite; although, cross-reactivity data for the *N*-oxide is generally similar for all reagents. In general, the cross-reactivity data obtained by ELISA confirms reported RIA data for the antiserum.

3.8. Calibration

The competitive ELISA was optimized for sensitivity, and calibration graphs were constructed for MAb 2B4D4 and the anti-ranitidine serum over the range 0.6 pg ml^{-1}–37 μg ml^{-1}. Fig. 5 clearly demonstrates that MAb 2B4D4 and the antiserum yield superimposable calibration graphs which suggests that the affinity constant for the MAb is similar to 1×10^9 l mol^{-1} reported for the antiserum.

4. Conclusions

A sensitive and specific monoclonal reagent antibody suitable for the quantitative determination of ranitidine has been developed using the innovative and rapid RIMMS technique. On comparing standard curves by competitive ELISA, the antibody afforded superimposable calibration graphs with the established polyclonal anti-ranitidine serum. In specificity studies, the monoclonal antibody appeared superior as it afforded lower cross-reactivity with the ranitidine sulphoxide metabolite.

This study clearly demonstrates that the RIMMS technique can be employed to produce high affinity monoclonal antibodies to drug haptens in $\sim$30 days, which represents a marked reduction compared to the time typically required by conventional means of hybridoma generation. Furthermore, RIMMS affords time-savings compared to polyclonal antiserum development as the ranitidine antiserum required $\sim$8.5 months to

produce. Consequently, RIMMS has a clear impact in allowing antibodies to be available earlier in drug development.

Since this study, the RIMMS technique has proved successful for the production of monoclonal antibodies to a broad range of antigens; including: another drug hapten [14] (GI198745, a candidate drug currently in clinical trials at Glaxo Wellcome), four peptide haptens [3], 12 recombinant proteins, cell extracts [15], and a protein expressed in vivo using DNA-based immunization [16].

The RIMMS technique, and the procedures described above should prove valuable to groups wishing to develop affinity-matured monoclonal antibodies to haptens or other classes of antigens.

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