Generation of Class-Selective Monoclonal Antibodies Against the Penicillin Group

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To develop a penicillin-specific ELISA, different attempts were made to obtain monoclonal antibodies specific for the common structure of penicillins. Ampicillin was coupled to different carrier proteins (bovine serum albumin, chicken ovalbumin, and thyroglobulin) to render it immunogenic. Different coupling methods were compared: two methods using a cross-linker (glutaraldehyde or a succinimide ester), one carbodiimide-mediated coupling method, and one method without any cross-linker or mediator molecule (physiological binding). Mice were immunized with the conjugates intraperitoneally or in the footpad. A screening ELISA was developed to detect anti-ampicillin antibodies in sera. Specificity and affinity of the antibodies were demonstrated by inhibiting their binding with a 10 mM solution of ampicillin. No difference could be observed using electrofusion or PEG-mediated fusion. For the production of the monoclonals, an intraveneous final boost gave antibodies with better specificity and affinity than an intraperitoneal final booster injection. At least one anti-ampicillin monoclonal antibody (19C9) cross-reacts with penicillin G, oxacillin, dicloxacillin, and carbenicillin, and not with sulfanilamide, chloramphenicol, neomycin, and streptomycin, and is therefore considered interesting for developing a penicillin-specific ELISA.

Keywords: *β-Lactam antibiotics; immunogenicity; enzyme-linked immunosorbent assay; monoclonal antibodies*

INTRODUCTION

Penicillins are widely used in veterinary medicine. As a result, food derived from animals treated with antibiotics may be contaminated with those drugs. To protect consumers from risks related to drug residues, maximum residue levels (MRL) are determined by law (EEC 2377/90). To analyze for the presence or absence of penicillin residues in meat and milk products, microbial inhibition tests and receptor assays are most commonly used (*1*, *2*, *3*). These methods either are not specific enough or do not allow detection of the whole group of penicillins. Physicochemical methods, such as high-performance liquid chromatography (HPLC), need time-consuming sample preparation, and only one sample can be handled at a time. An alternative for the restrictions inherent to these techniques is the detection of residues by enzyme-linked immunosorbent assays (ELISAs) (*4*). ELISAs are quick, specific, and sensitive, and they have the additional advantage of analyzing several samples simultaneously.

The aim of the present study was to develop an ELISA that is specific for the group of penicillin antibiotics. As β-lactam antibiotics share a 6-aminopenicillanic acid structure (a β-lactam ring structure coupled to a thiazolidin ring, Figure 1) and differ in their acyl-side chain, the strategy followed was to induce and select monoclonal antibodies directed against the common 6-aminopenicillanic acid core. Using ampicillin−protein conjugates, studies on the antigenicity of penicillins have indicated three important epitopes: the acyl-side chain, the common thiazolidin ring, and newly formed structures that arise by coupling penicillins to a carrier protein (*5*, *6*). In the present study, the immunogenicity in mice of different ampicillin conjugates was compared, and the characteristics of the produced ampicillin-specific monoclonal antibodies were determined.

MATERIALS AND METHODS

Reagents and Chemicals. The following were purchased from Sigma-Aldrich (Bornem, Belgium): benzylpenicillin, amoxicillin, 6-aminopenicillanic acid, bovine serum albumin (BSA), thyroblobulin, ovalbumin (OVA), glutaraldehyde, hydroxylamine, tetrahydrofuran, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimidemetho-*p*-toluenesulfonate (MEDC), *s*-acetylmercaptosuccinic anhydride (SAMSA), 3-maleimidobenzoic-*N*-hydroxisuccinimide ester (MBS), Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid), citraconic anhydride, bicinchoninic acid (BCA), copper(II)sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), and OPI supplement media. Oxacillin, dicloxacillin, and cloxacillin were obtained from ICN Biochemicals (Asse-Relegem, Belgium). Ampicillin, poly(ethylene glycol) 1500 (PEG), ABTS (2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate)) tablet, and ABTS buffer were obtained from Roche Diagnostics (Brussels, Belgium). The ABTS substrate solution was prepared by

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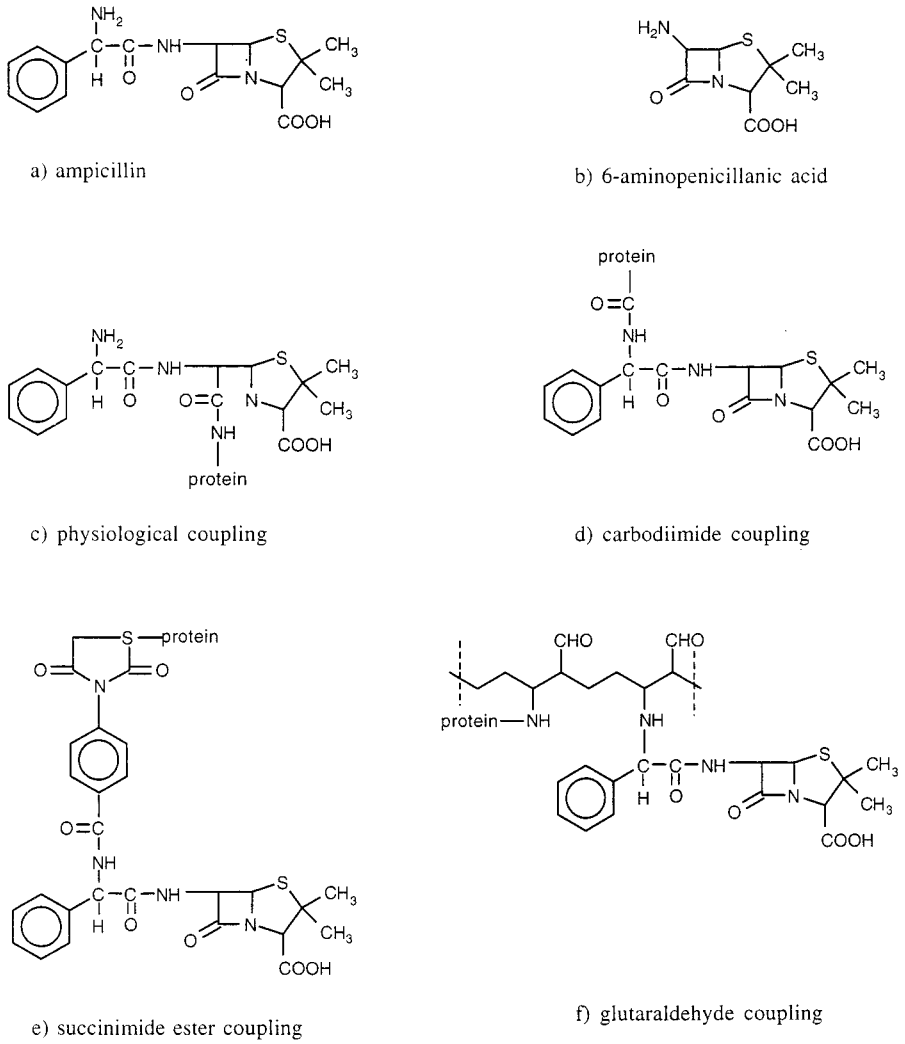

Figure 1. Chemical structures of ampicillin−protein conjugates using different coupling methods: (a) ampicillin; (b) 6-aminopenicillanic acid; (c) physiological binding; (d) carbodiimide-mediated coupling; (e) 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) coupling; (f) glutaraldehyde coupling.

dissolving 1 ABTS tablet (5 mg) in 50 mL of ABTS buffer. Complete Freund's adjuvant (CFA) and incomplete adjuvant (IFA) were provided by Difco Laboratories, Biotrading (Bierbeek, Belgium). Tween 20 (polyoxyethylene sorbitan monolaurate) was purchased from Merck-Belgolabo (Overijse, Belgium). Rabbit anti-mouse immunoglobulins conjugated to peroxidase (α-mHRP prosan, code n*o*. P0260) were obtained from DAKO Diagnostica, Prosan (Ghent, Belgium). Dialysis tube VIKING (12000−14000 MW cutoff) was provided by ROTH, Fiers (Kuurne, Belgium). ELISA microtiter plates (maxisorp) were obtained from NUNC, Life technologies (Merelbeke, Belgium). Tissue culture plates were obtained from Greiner (Wemmel, Belgium). Dulbecco modified Eagle's medium (DMEM), glutamine, gentamycin, sodium pyruvate, foetal calf serum (FCS), and hypoxanthine, aminopterine, and thymidine supplement (HAT) were purchased from GibcoBRL, Life technologies (Merelbeke, Belgium). All other chemicals were of reagent grade or better. HAT-selection medium consisted of DMEM containing 20% FCS, 1% glutamine, 0,1% gentamycin, 1% sodium pyruvate, 1% OPI supplement media, and 2% HAT. Phosphate buffered saline (PBS) solution (0.15 M, pH 7.4) was prepared by dissolving 0.8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 mL of distilled H_2O. The pH was adjusted to 7.4 with addition of NaOH or HCl. Finally, the solution was made up to 1 L with distilled H_2O.

Carbodiimide-Mediated Penicillin−Carrier Conjugation (*7*). To protect the amino groups of the carrier protein from reacting with carbodiimide, the amino groups were blocked with citraconic anhydride. The carrier protein and citraconic anhydride were dissolved in 4 mL of distilled H_2O in a molar ratio of 1:10, respectively. The pH was then adjusted to 8.5 with 1 M NaOH. After 15 min incubation at RT, 1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimidemetho-*p*-toluenesulfonate (MEDC) dissolved in 200 μL of distilled H_2O, was added in a molar ratio of protein/MEDC of 1:10. After 5 min incubation at RT, ampicillin was added in a molar ratio of protein/amp of 1:100 for coupling to albumines, and in a molar ratio of 1:1000 for coupling to thyroglobulin. The mixture was then incubated for 2 h at RT. Deprotection of the amino groups of the carrier protein was done by dialysis (12000−14000 cutoff) against 1 L of 5% acetic acid for 3 h at 4 °C. Finally, the product was dialyzed (12000−14000 cutoff) for 24 h at 4 °C against 3 changes of 1 L of PBS. Aliquots of the conjugates carbo amp−BSA, carbo amp−OVA, and carbo amp−thyro were stored at −20 °C.

Preparation of Penicillin−Succinimide Ester−Carrier Conjugate (*8, 9*). *(a) Introduction of Sulfhydryl Groups on the Carrier Protein (Acetylthio-Carrier Protein).* *s*-Acetylmercaptosuccinic anhydride (SAMSA, 4.08 mmol) was added slowly to 0.077 mmol carrier protein dissolved in 15 mL of 0.1 M potassium phosphate buffer pH 7.3. By doing this, the pH was maintained between 7 and 7.5. Once all of the SAMSA was added, the pH was lowered to 6 by adding 1 N HCl. The solution was dialyzed during 1 week against distilled H_2O, whereafter the conjugate was lyophilized.

(b) Removal of the Acetyl Group of Acetylthio−Carrier Protein. Deoxygenated 0.1 M hydroxylamine (10 μL) was added

to 20 mg of lyophilized acetylthio−carrier protein dissolved in 500 μL of deoxygenated 0.1 M phosphate buffer, pH 7.3. The solution was then mixed under N_2 until no further increase in number of sulfhydryl groups could be observed. The number of sulfhydryl groups was determined using the Ellman standard method (*10*). Ellman's reagent (5,5′-dithiobis(2-benzoic acid), 20 μL) was added together with 20 μL of sample to 1 mL of sodium phosphate buffer 0.05 M pH 8 and incubated for 15 min at room temperature. The reaction between Ellman's reagent and free sulfhydryl groups results in a yellow color which is measured spectrophotometrically at 412 nm.

(c) 3-Maleimidobenzoic-*N*-hydroxisuccinimide ester (0.015 mmol MBS) dissolved in 0.5 mL of tetrahydrofuran was added to 0.015 mmol ampicillin dissolved in 1 mL of 0.05 M sodium phosphate buffer pH 7. The mixture was then incubated during 1 h with gentle stirring. Subsequently, tetrahydrofuran was removed by mixing the solution under N_2. The excess of MBS was removed by extraction with 3 × 5 mL of methylene-chloride/ether (1:2; v/v). The aqueous phase contained the MBS-coupled ampicillin (amp−MBS) and was used in the next step.

(d) The thio−carrier-protein solution was added to the amp−MBS solution and incubated for 2 h at 25 °C. The mixture was then dialyzed (12000−14000 cutoff) against PBS during 3 days. Aliquots of the conjugates amp−MBS−OVA and amp−MBS−BSA were stored at −20 °C.

The coupling efficiency was established by determining the number of sulfhydryl groups left after coupling (*10*) and subtracting the amount from the number determined in step (b). As only one ampicillin molecule can be coupled to one sulfhydryl group, the amount of bound ampicillin molecules equals the amount of reacted sulfhydryl groups:

$$\text{number of ampicillin molecules} = [(\text{Ev} - \text{En})/\text{Em}] \times f \times N_A$$

where Ev = absorbance at 412 nm of the thio-carrier solution after reaction with Ellman's reagent, En = absorbance at 412 nm of the final product after reaction with Ellman's reagent, Em = molar extinction coefficient for the Ellman's reagent at 412 nm (13600), N_A = number of Avogadro, and f = dilution factor.

The amount of carrier protein molecules was determined by measuring the protein concentration of the thio−carrier-protein solution via the absorbance at 280 nm before adding amp−MBS. The coupling efficiency is expressed as the number of ampicillin molecules bound to one carrier molecule in the final product (efficiency = number of ampicillin molecules/carrier molecule).

Preparation of Penicillin−Glutaraldehyde−Carrier Conjugate (*7, 11*). Ampicillin (0.4 mmol) dissolved in 8 mL of dimethylformamide was added to 0.003 mmol carrier protein dissolved in 16 mL of phosphate buffered saline 0.15 M pH 7.4 (PBS). Subsequently, 0.15 mL of glutaraldehyde (25%) was added dropwise to the solution. After 3 h of gentle stirring at RT, the reaction mixture was dialyzed (12000−14000 cutoff) against PBS during 3 days. Aliquots of the conjugates amp−glut−BSA and amp−glut−OVA were stored at −20 °C.

To define the coupling efficiency, the amount of carrier molecules had to be determined first by measuring the protein concentration of the final product via its absorbance at 280 nm (penicillin shows no absorbance at 280 nm). In a second step, the amount of bound penicillin in the final product was determined using the BCA method (*12*): 1 mL of BCA reagent (50 parts BCA + 1 part $CuSO_4 \cdot 5H_2O$) is added to 50 μL of the sample whereafter the absorbance is measured at 562 nm after 30 min incubation at 37 °C. Because the carrier protein in the final product also reacts with BCA reagent, a sample containing only the carrier protein at a concentration equal to the one that was determined in step 1 was also used to react with BCA reagent. The absorbance of penicillin in the final product equals the absorbance of the final product minus the absorbance of the sample with the carrier molecule at a concentration equal to the concentration determined in step 1. A calibration curve for ampicillin was established by plotting the concentration of a standard dilution of ampicillin against the absorbance at 562 nm obtained for those standard samples after reaction with BCA reagent. Extrapolation of the absorbance of penicillin in the final product led to the concentration of penicillin in the product, and consequently to the amount of ampicillin molecules.

The efficiency of the reaction is expressed as the number of ampicillin molecules bound to one carrier molecule in the final product.

Preparation of Physiological Penicillin−Carrier Conjugate (*13*). One gram of ampicillin and 0.25 g of carrier protein were dissolved in 10 mL of 0.008 M diethylbarbituric acid pH 8.5 and incubated for 24 h at 37 °C. Subsequently, the solution was dialyzed against PBS for 2 days. Aliquots of the conjugate phys−amp−OVA and phys−amp−BSA were stored at −20 °C. The coupling efficiency was determined as done for the glutaraldehyde conjugates.

Immunization and Production of Monoclonal Antibodies (Mab). Balb/c mice (minimum 10 weeks old) were immunized with different ampicillin−protein conjugates using 3 different immunization procedures. The first method consisted of an intraperitoneal injection of 50 μg of conjugate (emulsified in 100 μL of sterile PBS and 100 μL of CFA), followed 3 weeks later by a second (and third) intraperitoneal injection with the same amount of conjugate (emulsified in sterile PBS and IFA). The second method differed from the first method in that the second (and the third) injection was given after 4 weeks. In both methods, four or 5 days before fusion, the mice with the highest specific serum antibodies were injected intraperitoneally with 100 μg of conjugate in PBS. Blood samples were collected from the tail vein of the mice starting two weeks after the second immunization and tested in ELISA for the presence of penicillin-specific antibodies. In the third method both hind footpads of mice were injected with 100 μg of conjugate (emulsified sterile PBS and IFA). Two weeks after this single injection the popliteal lymph nodes were used for fusion.

Hybridomas were obtained by poly(ethylene glycol) mediated fusion or by electrofusion of $SP_2OAG/14$ mouse myeloma cells with either spleen (method 1 and 2) or lymph node (footpad immunization) cells from immunized mice. The isolation of lymphocytes, culturing of the myeloma cells, and the poly(ethylene glycol) mediated fusion were performed according to the procedures previously described by Harlow and Lane (*14*). For the electrofusion, the myeloma cells and the lymphocytes were mixed at a ratio of 1:1, washed by centrifugation (5 min, 800*g*, 18 °C), and resuspended in 300 μL of sucrose-containing buffer (0.255 M sucrose, 0.2 mM $CaCl_2$, and 0.2 mM $MgCl_2$ diluted in distilled water). The suspension was transferred into the fusion chamber of the electroporesis apparatus (ICN Biomedicals, Asse-Relegem, Belgium). The fusion was performed by dielectrophoresis (frequency 1 MHz, field strengh 15 V/0.5 cm, 30 s), electroporation (field strength 0.15 V/0.5 mm, 5 μs, one pulse), and dielectrophoresis again. Finally, the cell suspension was removed from the fusion chamber, diluted in HAT-selection medium and distributed over 96-well tissue culture plates at 1000 to 10000 cells per well. Hybridomas producing penicillin-specific antibodies were cloned twice by limiting dilution (*14*).

Indirect Antibody ELISA. Microtiter plates were coated overnight at 4 °C with ampicillin−succinimide ester−ovalbumin conjugate (amp−MBS−OVA; 100 μL/well) diluted in bicarbonate coating buffer (0.05 M; pH 9.4). The plates were washed 3 times with PBS containing 0.05% Tween 20 between each incubation step. Free binding sites were blocked with 200 μL of a glycine solution (5% glycine in coating buffer) for 2 h at 37 °C. Subsequently, 100 μL/well of an appropriate dilution of mice sera or hybridoma supernatant in PBS containing 3% BSA and 0.05% Tween 20 was added. The plates were incubated for 1 h at 37 °C. Then, 100 μL/well of the secondary antibody (diluted in PBS containing 3% BSA and 0.05% Tween 20) was e added for 1 h at 37 °C, whereafter 50 μL/well enzyme substrate ABTS solution was added. Subsequently, the plates

Table 1. Methods Used for Ampicillin−Protein Conjugation

conjugation method	coupling efficiency ($molec_{ampicillin}$/$molec_{carrier}$)	determination method
carbodiimide	ND[a]	
glutaraldehyde	8.5−16	BCA
succinimide ester	8−13	Ellman standard method
physiological	11−13	BCA

[a] ND, not determined.

were incubated at 37 °C. The color development was measured at 405 nm using an ELISA reader (Spectrafluor, TECAN) and presented as optical density (OD).

Competitive Inhibition ELISA. The competitive inhibition ELISA and the indirect antibody ELISA were performed in the same manner with the only difference being that in the former procedure the samples (mice sera and hybridoma supernatant) were preincubated at RT with a 10 mM ampicillin solution or with serial dilutions (concentrations ranging from 10 mM to 0.01 mM) of a β-lactam antibiotic mixture containing ampicillin, benzylpenicillin, carbenicillin, dicloxacillin and oxacillin. After 1 h the antibody−antibiotic mixture was tested in ELISA.

The competition in the ELISA between a free penicillin in the sample and the coated ampicillin (amp−MBS−OVA) was calculated with the formula:

$$\text{competition (\%)} = (1 - (A/A_o)) \times 100$$

with A = absorbance of a tested sample solution and A_o represeting the absorbance of a similar solution without penicillin.

RESULTS

Preparation of Penicillin Immunogens. Four coupling procedures were used to develop penicillin−carrier conjugates: the carbodiimide-mediated coupling, glutaraldehyde, the succinimide ester method, and a physiological reaction method. Ampicillin was chosen as hapten and conjugated to bovine serum albumin (BSA), ovalbumin (OVA), or thyroglobulin (thyro) using the four procedures (Table 1). Conjugation was performed in such a way that the common structure of penicillins, the thiazolidin ring, was left unchanged for the induction of common antibodies to β-lactam antibiotics. The chemical structures of ampicillin, 6-aminopenicillanic acid, and the obtained immunogens were presented earlier in Figure 1. The efficiency of the coupling reaction was determined as the amount of ampicillin molecules bound to one carrier molecule. For each procedure, a coupling efficiency of approximately 10 (8 to 16) was obtained (Table 1), except for the carbodiimide where coupling efficiency could not be determined.

Antibody Response. Blood samples were collected 2 weeks after each immunization and were tested for anti-ampicillin antibodies in the indirect and the inhibition ELISA (Table 2). No anti-ampicillin antibodies could be detected in the mice immunized with the carbodiimide-mediated ampicillin−albumin conjugates. Moderate antibody responses against ampicillin were obtained after immunization with the carbodiimide-mediated ampicillin−thyroglobulin, the glutaraldehyde, or succinimide conjugates. Subsequent immunizations did not enhance the response. A high antibody response was induced using the physiological ampicillin−protein conjugate. Using OVA or BSA as the carrier protein, no difference in antibody response against the hapten could be observed when comparing immunogens differing only for their carrier protein.

Production of Mab. An overview of the fusion experiments is given in Table 3 for mouse 4 immunized intraperitoneally (IP) with carbo amp−thyro, mouse 15 immunized IP with amp−glut−BSA, mouse 24 immunized IP with amp−MBS−BSA, mouse 35 immunized in the footpad (FP) with phys−amp−OVA, and mouse 36 immunized FP with amp−MBS−OVA. The footpad procedure resulted in the collection of only 1 to 2×10^7 cells from the popliteal lymph nodes and in the generation of few hybridomas, of which two had low affinity for penicillin: more than 10 mM ampicillin was needed to obtain 50% competition in the inhibition ELISA (Table 3). The IP immunization with carbodiimide-mediated thyroglobuline conjugate, glutaraldehyde, or succinimide conjugates followed by fusion of spleen lymphocytes resulted in at least 10 times higher number of hybridomas. However, again, none or only a few of the hybridomas produced penicillin-specific antibodies which had, moreover, low affinities as presented in Table 3 for mice 4, 15, and 24.

Four mice immunized intraperitoneally with a physiological conjugate were selected for spleen cell fusion experiments (mouse numbers 161−164, Tables 4 and 5). This selection was based upon either a high serum antibody titer (mice 163 and 164) in the indirect ELISA, or a strong competition effect in the competitive inhibition ELISA (mice 161 and 162).

The immunization frequency, the injection method at the final booster injection, the detection limit of diluted

Table 2. Immunogenicity of Different Ampicillin−Protein Conjugates

conjugation method	carrier protein[a]	immunizations: route[b]	immunizations: no. of mice	no. of mice with specific response in indirect ELISA	indirect ELISA[c]	inhibition ELISA[d]
carbodiimide	BSA	IP1	3	0	−	ND[e]
	OVA	IP1	3	0	−	ND
	thyro	IP1	3	3	+/+++	ND
glutaraldehyde	BSA	IP1	3	3	+/+++	0/++
	OVA	IP1	3	3	+/+++	0/++
succinimide	BSA	IP1	3	3	++	+/++
physiological	BSA	IP2	4	4	++++	++++

[a] BSA, bovine serum albumin; OVA, chicken egg albumin; thyro, thyroglobulin. [b] IP1, intraperitoneal, interval between subsequent immunization = 3 weeks; IP2, intraperitoneal, interval between subsequent immunization = 4 weeks. [c] Scores are given according to OD levels obtained with 1/20 diluted sera: −, < 0.200; +, 0.200−0.500; ++, 0.500−1.000; +++, 1.000−1.800; ++++, >1.800. [d] Scores are given according to the obtained inhibition of antibody binding by preincubation with 10 mM ampicillin: +, < 50%; ++, 50−75%; +++, 75−90%; ++++, >90%. The percentage of inhibition = 100 − [OD value of inhibition ELISA/OD value of indirect ELISA] × 100). [e] ND, not detected.

Table 3. Overview of the Data Obtained after Fusion Experiments

mouse no.	immunogen	route[a]	fusion procedure	number of hybridomas	number positives indirect ELISA	number positives[b] inhibition ELISA
4	carbo Amp−thyro	IP	PEG-mediated	313	48	0
15	amp−glut−BSA	IP	PEG-mediated	81	35	1
24	amp−MBS−BSA	IP	electrofusion	214	2	1
35	phys−amp−OVA	IFP	electrofusion	8	2	2
36	amp−MBS−OVA	IFP	PEG-mediated	4	0	0

[a] IP1, intraperitoneal, interval between subsequent immunization = 3 weeks; IFP, footpad. [2] Binding of antibodies to the coated ampicillin−carrier conjugate could be partially inhibited by preincubation with a 10 mM ampicillin solution (= 3.71 mg/mL).

Table 4. Analysis of Sera of Mice Immunized Intraperitoneally with the Physiological Conjugate

mouse no.	number of immunizations	final booster[a]	antibody titer in indirect ELISA	inhibition ELISA[b]
161	2	IP	1000	++
162	2	IP	1000	++++
163	5	IP	8000	+
164	4	IV	10000	+

[a] IP, intraperitoneal; IV, intraveneous. [b] Scores are given according to the obtained inhibition of antibody binding by preincubation with 10 mM ampicillin: +, < 50%; ++, 50−75%; +++, 75−90%; ++++, >90%. The percentage of inhibition = 100 − [OD value inhibition ELISA/OD value indirect ELISA] × 100.

Table 5. Analysis of Hybridomas of Mice Immunized with the Physiological Conjugate

mouse no.	total number	number positives in indirect ELISA	number positives[a] in inhibition ELISA	number with inhibition > 70%
161	> 3000	95	15	0
162	> 4000	90	37	(7)
163	± 3000	321	10	0
164	1417	180	32	21

[a] Binding of antibodies to the coated ampicillin−carrier conjugate could be inhibited partially or completely by preincubation with a 10 mM (3.71 mg/mL) ampicillin solution.

Table 6. Characteristics of the Selected Mab, Derived from Mice 161 and 164

			% inhibition[a] with ampicillin			cross-reaction[b] with 500 ng/mL		
mouse no.	Mab	isotype	5 mg/mL	100 μg/mL	100 ng/mL	pen. G	carb	oxa
161	12F6	IgM	22	10	0	−	−	−
161	12F5	IgM	20	11	0	−	−	−
161	13B2	IgM	26	12	0	−	−	−
164	10E5	IgG1	100	100	10	+	+	+
164	19C9	IgG1	100	100	13	+	+	+
164	10C2	IgG2a	100	100	0	+	+	+
164	23D12	IgM	100	75	0	−	+	+
164	2G4	IgG2a	100	75	0	+	−	−
164	9H3G3	NT[c]	100	78	0	−	−	−

[a] % inhibition was determined in the inhibition ELISA. Therefore, the monoclonal was preincubated during 30 min. with native ampicillin. [b] Cross-reactivity was examined in the inhibition ELISA. pen. G, penicillin G; carb, carbenicillin; oxa, oxacillin [c] NT, not tested

serum in the indirect ELISA, and the competition effect are represented in Table 4, and data obtained after cell fusions are given in Tables 5 and 6. Fusion experiments with mouse numbers 162 and 164 resulted in several hybridomas showing strong competition effects (Table 5). Unfortunately, the hybridomas derived from mouse 162 were not stable.

The most important finding was that the best hybridomas were obtained after spleen cell fusion of a mouse (mouse 164) with an intraveneous final booster, whereas the other mice got an intraperitoneal final booster injection.

Hybridomas Selected for Further Study. After subcloning and ascites production, the isotype and cross-reactivity with other penicillins were investigated. In Table 6 these characteristics are represented for Mabs derived from two mice: mouse 161 and mouse 164. As can be seen, Mabs derived from mouse 164 showed a higher percentage of inhibition than the Mabs derived from mouse 161. Binding of two of the Mabs (10E5, 19C9) of mouse 164 to an ampicillin−carrier conjugate could even be partially inhibited by preincubation with ampicillin at a concentration of 100 ng/mL. Results, therefore, indicate that the affinity of the antibodies from mouse 164 for native ampicillin is high. Furthermore, 3 of them (19C9, 10E5, and 10C2) cross-reacted with other penicillins, suggesting that they recognized common epitopes.

For the determination of cross-reactivity of the Mab 19C9 for several penicillins at their MRL value (Table 7), the competitive inhibition ELISA was performed at two different incubation temperatures (4 and 37 °C) and two different incubation times (30 and 60 min). The best results for the detection of ampicillin and penicillin G were obtained when the ELISA was incubated during 30 min at 4 °C. Otherwise, the detection of oxacillin, carbenicillin, and dicloxacillin was more sensitive at 37 °C. Cross-reaction of this antibody with sulfanilamide, chloramphenicol, neomycin, and streptomycin was not observed.

Table 7. Cross-Reactivity (at Maximum Residue Level, MRL) of Mab 19C9 with Other Penicillins and Some Other Antibiotics and Sulfanilamide, Measured in the Inhibition ELISA

molecule	concentration[a] (ng/mL)	% inhibition	MRL detection level?	incubation conditions on ELISA-plate
ampicillin	50	31	yes	4 °C, 30 min
penicillin G	50	25	yes	4 °C, 30 min
carbenicillin	50	25	yes	37 °C, 15 min
oxacillin	300	40	yes	37 °C, 60 min
dicloxacillin	300	40	yes	37 °C, 60 min
sulfanilamide	500	0		4 and 37 °C, 15 and 30 min
chloramphenicol	500	0		4 and 37 °C, 15 and 30 min
neomycin	500	0		4 and 37 °C, 15 and 30 min
streptomycin	500	0		4 and 37 °C, 15 and 30 min

[a] MRL, maximum residue level.

DISCUSSION

The carrier−hapten conjugates used for immunization in the present study were characterized by determining the number of ampicillin molecules per carrier molecule and by their capacity to induce polyclonal antibody responses in mice. For the carbodiimide mediated conjugates, no appropriate method was available for determining the number of ampicillin molecules per carrier molecule. For the other conjugates, the number of ampicillin molecules coupled to one molecule of protein could be measured, and was similar to or slightly higher than those obtained by other investigators (*7*, *11*, *13*). Katsutani and Shionoya (*13*) constructed physiological benzylpenicillin−BSA and −OVA conjugates with a hapten/carrier coupling efficiency of 18/1 and 10/1, respectively. Märtlbauer (*11*) used the glutaraldehyde method to obtain sulfonamide−carrier conjugates with a coupling efficiency of 7/1, and the active ester method (succinimide) for coupling natamycin to a carrier with a coupling efficiency of 5/1. Van Regenmortel et al. (*7*) stated that a coupling efficiency of 5 to 20 mol hapten per mol carrier was high enough to render the hapten immunogenic. This was consistent with findings in the present study, as most immunizations induced antibody responses. However, the antibody response against nonphysiological conjugates was moderate to low. Also, Usleber et al. (*15*) immunized with ampicillin coupled to different carriers using glutaraldehyde as cross-linker and found the conjugates to be non- or weakly immunogenic.

Immunizations with the carbodiimide-mediated ampicillin−albumin conjugates did not induce anti-ampicillin antibodies, whereas antibodies against the carrier protein could be demonstrated. However, when thyroglobulin was used as the carrier protein, an ampicillin-specific response was induced. Because thyroglobulin is at least 10 times larger than albumin, the lack of antibody response to the carbodiimide-mediated ampicillin−albumin conjugate suggests that albumin did not carry as many ampicillin molecules as thyroglobulin.

Using the immunogens constructed with cross-linker or the carbodiimide-mediated conjugate, few hybridomas were obtained. Only some of them produced low-affinity penicillin-specific antibodies (Table 3). This indicates that those immunogens or the immunization procedures were unfavorable. Immunization using the footpad method resulted in few hybridomas compared to spleen cell fusion experiments. Consequently, a lower number of penicillin specific hybridomas were obtained (mice 35 and 36, Table 3). Interestingly, the two hybridomas of mouse 35, which were found positive in the indirect ELISA, were also positive in the inhibition ELISA. Fusion experiments following intraperitoneal immunization always resulted in a higher amount of hybridomas, but only very few were found positive in the inhibition ELISA (Table 3, mouse numbers 4, 15, and 24). Mirza et al. (*16*) used human insulin as immunogen to compare different routes of immunization for hybridoma production. They found that footpad immunization followed by popliteal lymph node lymphocyte fusion yielded 100% of the hybridomas secreting a specific antibody, compared to subcutaneous or intraperitoneal immunization followed by splenic lymphocyte fusion (8%). However, another study using the footpad immunization procedure for the production of antibodies against viral and bacterial antigens, resulted in 6 to 28% hybridomas secreting specific antibodies *(17)*. A disadvantage of the footpad immunization is the low number of lymphocytes that can be used for cell fusion. Furthermore, this kind of immunization is very painful and should therefore be used only if really necessary (*14*).

In this study two methods were used for fusion: electrofusion and poly(ethylene glycol) (PEG)-mediated fusion. Electrofusion is widely described to yield a higher fusion efficiency with more antigen-specific hybridomas than the PEG-mediated fusion (*18*, *19*, *14*). However, in the present study no difference could be observed between the two methods.

A high antibody response was obtained against the physiological ampicillin−protein conjugates. Similar conjugates are formed in vivo following penicillin administration (*13*). Natural conjugation results in an open β-lactam ring structure with loss of antimicrobial activity, but not immunogenicity (*20*). The Mabs obtained from mice immunized with physiological conjugates showed strong competition in the inhibition ELISA. The antibodies could be partially inhibited for binding to the coated ampicillin−carrier conjugate after preincubation of those antibodies with ampicillin at a concentration of 50 ng/mL.

From the results it appears that the route of antigen administration during the final booster injection could be very important. The Mabs derived from mouse 164, which was boosted intraveneously, recognize penicillin better than those obtained from mouse 161, which was boosted intraperitoneally. Intraveneous injection will result in a rapid and strong response of splenic lymphocytes, as the antigen will be captured quickly in the spleen (*14*). So, directing the antigen during the final boost toward the lymphocytes that will be used in the fusion, seems to increase the number of antigen-specific antibodies. Mab 19C9 displayed a specific cross-reactivity, as defined in the inhibition ELISA, with ampicillin, penicillin G, oxacillin, dicloxacillin, and carbenicillin, and not with sulfanilamide, chloramphenicol, neomycin, and streptomycin. Consequently, 19C9 appears to be a

β-lactam-specific antibody and looks very promising for developing an ELISA that will be able to detect most penicillins at their MRL concentrations.

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