Ceftiofur Sodium: Monoclonal Antibody Development and Cross-Reactivity Studies with Structurally Related Cephalosporins

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Monoclonal antibodies were prepared against ceftiofur using its hydrolyzed form, desfuroylceftiofur, as the hapten. Desfuroylceftiofur was prepared *in situ* and conjugated directly to maleimide-activated carrier proteins bovine serum albumin and keyhole limpet hemocyanin (KLH). Balb/c mice were immunized with the desfuroylceftiofur−KLH conjugate (KLH-SMCC−desCef). Splenic lymphocytes from these mice were fused with SP2/0 myeloma cells to produce hybridomas. Two stable monoclonal antibodies, Cef-68 and Cef-116, of subclasses IgG_{2a} and IgG_1, respectively, were isolated. An indirect competitive inhibition enzyme-linked immunosorbent assay (ciELISA) was developed for the quantitative detection of ceftiofur in liquid samples using a heterologous assay system to provide a more sensitive ELISA. Detection limits for ceftiofur were 32 and 0.3 ppb for the two clones Cef-68 and Cef-116, respectively. The ciELISA described here provides a sensitive assay for the detection of ceftiofur. Cross-reactivity studies with a variety of related cephalosporins and penicillins indicated that only a limited number of the cephalosporins and none of the penicillins that were tested competed in the ciELISA.

Keywords: *Ceftiofur; immunoassay; monoclonal antibody; residue analysis*

INTRODUCTION

Ceftiofur is an FDA-approved veterinary cephalosporin for the treatment of respiratory diseases in cattle, horses, and swine (Food and Drug Administration, 1988, 1991, 1992). In a recent paper we discussed the development of polyclonal antibodies and the influence of hapten design on the nature of the resulting murine polyclonal antibodies (Rose *et al.*, 1995). In addition, we studied the effects of various plate-coating antigens on assay sensitivity. Current methods for the detection of ceftiofur include HPLC (Beconi-Barker *et al.*, 1995; Jaglan *et al.*, 1989, 1990, 1992, 1994; Gilbertson *et al.*, 1995; Owens *et al.*, 1990; Tyczkowska *et al.*, 1993; Halstead *et al.*, 1992; Mahrt *et al.*, 1992; Cervantes *et al.*, 1993), and microbiological assays (Jaglan *et al.*, 1992, 1994; Gilbertson *et al.*, 1990; Louhi *et al.*, 1992; Meyer *et al.*, 1992; Owens *et al.*, 1990; Soback *et al.*, 1991; Yancey *et al.*, 1987). While accurate, these methods are time-consuming and require expensive instrumentation. Detection methods that are specific and deliver faster results without the use of expensive, sophisticated equipment are desirable. Therefore, we chose to develop a monoclonal antibody (MAb)-based immunoassay for ceftiofur to provide a less expensive and less cumbersome alternative to those methods indicated above. We describe the production and characterization of two hybridoma cell lines that produce monoclonal antibodies specific for ceftiofur. These MAbs have been formatted into a competitive indirect enzyme-linked immunosorbent assay (ciELISA). We also describe the cross-reactivity of the MAbs with related cephalosporins and penicillins using this ciELISA.

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MATERIALS AND METHODS

Reagents and Equipment. Ceftiofur sodium was kindly donated by The Upjohn Co. (Kalamazoo, MI). Cefteram sodium and ceftriaxone sodium were kindly donated by Hoffmann-La Roche Inc. (Nutley, NJ). Cefuroxime sodium and ceftazidime pentahydrate were kindly donated by Glaxo Manufacturing Services Ltd. (Barnard Castle, County Durham, England). RIBI adjuvant was purchased from RIBI ImmunoChem Research, Inc. (Hamilton, MT). Bovine serum albumin (BSA), ovalbumin (OVA), goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (IgG−peroxidase), polyoxyethylene sorbitan monolaurate (Tween 20), hypoxanthine, aminopterin, thymidine, poly(ethylene glycol) (PEG 4000), amoxicillin, ampicillin sodium, cefaclor, cefadroxil, cefamandole sodium, cefazolin sodium, cefoperazone sodium, cefotaxime sodium, cefoxitin sodium, cefsulodin sodium, cephalothin sodium, cephapirin sodium, cephradine, cloxacillin sodium, and penicillin G sodium were purchased from Sigma Chemical Co. (St. Louis, MO). 2,6,10,14-Tetramethylpentadecane (pristane) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Iscove's media, fetal bovine serum (FBS), and penicillin/streptomycin solution were obtained from Gibco (Grand Island, NY). Keyhole limpet hemocyanin (KLH), maleimide-activated BSA (BSA−SMCC), and maleimide-activated KLH (KLH-SMCC) were purchased from Pierce (Rockford, IL). Nunc-Immunoplates, Maxisorp F96, were purchased from PGC Scientifics (Gaithersburg, MD). K-Blue substrate was purchased from Elisa Technologies (Lexington, KY). The antibody isotyping ELISA kit was obtained from Fisher (Philadelphia, PA). Thin-layer chromatography was performed on precoated silica gel 60 F_{254} plates (0.2 mm) from Riedel de Häen (Gibbstown, NJ). Balb/c mice were obtained from Harlan−Sprague−Dawley (Houston, TX). A Bio-Rad Model 3550 microplate reader with software version 4.3 and reader-driver software 1.0 application program were obtained from Bio-Rad Laboratories (Hercules, CA).

Hapten−Protein Conjugates. Two conjugation methods were used to prepare hapten−protein conjugates as described earlier (Rose *et al.*, 1995). Briefly, ceftiofur was hydrolyzed to produce the desfuroylceftiofur metabolite *in situ*. Desfuroylceftiofur (desCef) containing a free thiol group was then conjugated to BSA-SMCC and KLH-SMCC to produce the

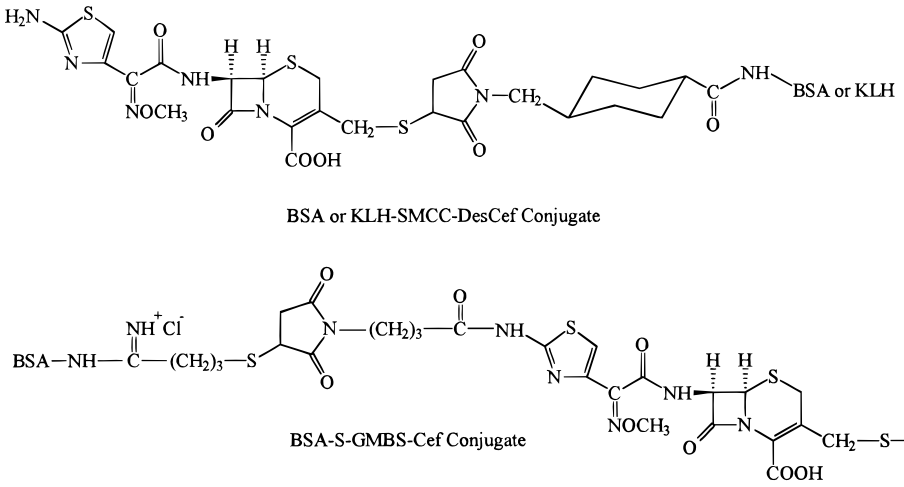

Figure 1. Structures of desfuroylceftiofur conjugated via the thiol to maleimide-activated carrier proteins to give the BSA-SMCC−desCef and KLH-SMCC−desCef conjugates and the structure of ceftiofur coupled via the amine group to the heterobifunctional cross-linking reagent, GMBS, and then conjugated to thiolated BSA to give the BSA-*s*-GMBS−Cef conjugate.

conjugates BSA-SMCC−desCef and KLH-SMCC−desCef, respectively. KLH-SMCC−desCef was used as the immunogen, and BSA-SMCC−desCef was used as the plate-coating antigen in initial screening experiments. The evaluation of the ciELISAs was performed using a heterologous plate-coating antigen, BSA-*s*-GMBS−Cef, instead of BSA−SMCC−desCef, as described earlier (Rose *et al.*, 1995.). In this conjugation the free amine on ceftiofur reacts with the succinimide moiety of the heterobifunctional cross-linking reagent, GMBS. The maleimide moiety of the hapten cross-linker is coupled to sulfhydryl groups on thiolated carrier proteins. The structures of the various haptens are shown in Figure 1.

Immunization Protocol. Female Balb/c mice were immunized intraperitoneally on day 1 with 100 μg of KLH-SMCC−desCef in a 0.2 mL solution of physiological saline and RIBI adjuvant (prepared as suggested by the manufacturer), and boosters were given on days 14, 28, and 43. Mice were bled through the tail vein on day 50, and serum titers were determined by ELISA using BSA-*s*-GMBS−Cef as the plate-coating antigen. On day 145, one mouse was boosted with a 100 μg dose of the immunogen prepared in physiological saline (no RIBI adjuvant), and 4 days later its spleen was removed and used for the production of hybridomas.

Hybridoma Production. Hybridomas were produced following fusion of SP2/0 myeloma cells with splenocytes from immunized mice as previously described (Stanker *et al.*, 1986). The fused cells were resuspended in hypoxanthine−aminopterin−thymidine (HAT) medium and plated onto 30 macrophage-containing 96-well microculture plates and were incubated at 37 °C in a 5% CO_2 atmosphere; subsequently, supernatants were screened for antibodies against ceftiofur by the ELISA method at 10 days following the fusion. Hybridoma cells that produced monoclonal antibodies capable of being inhibited by ceftiofur were cloned at least twice by limiting dilution.

Antibodies and ELISA Methods. A standard ELISA method in which the hapten conjugate is immobilized on 96-well microtiter plates was used to evaluate the response of Balb/c mice toward the immunogen KLH-SMCC−desCef. The microtiter immunoplates were washed three times with a solution of 0.05% (v/v) Tween 20 in deionized water (Tween 20−water) and then coated with 100 μL of the coating antigen, BSA-SMCC−desCef (100 ng/well), in deionized water. The plates were dried overnight at 37 °C. The plates were then washed three times with PBS-9 (0.01 M sodium phosphate, 0.15 M sodium chloride, pH 9), and the unbound active sites were blocked with 200 μL of 3% (w/v) nonfat milk in PBS-9 for 30 min at 37 °C. The plates were washed three times with PBS-9, and 100 μL of supernatant containing the antibody was added to the microtiter plates, which were subsequently incubated for 60 min at 37 °C. The unbound antibody was removed by washing the plate five times with Tween 20−water. Next, 50 μL of goat anti-mouse IgG−peroxidase conjugate (1:500 dilution) was added, followed by incubation of the plates at 37 °C for 60 min. Finally, the plates were washed five times with Tween 20−water, 100 μL of K-blue substrate was added, and the absorbance was read at 655 nm. For the ciELISA, the plates were prepared as above except that BSA-*s*-GMBS−Cef was used as the plate-coating antigen. Competitors were prepared in assay buffer [0.1 M Tris, 0.15-M sodium chloride, 0.01% (w/v) nonfat milk in deionized water, pH 7.75], added to the wells, and diluted in a 2-fold fashion immediately prior to addition of the anti-ceftiofur MAb. Tissue culture media containing the anti-ceftiofur MAbs were used at final dilutions of 1:20 and 1:100 for MAbs Cef-68 and Cef-116, respectively. The following competitors were used in a cross-reactivity study: ceftiofur sodium, cefteram sodium, ceftriaxone sodium, cefuroxime sodium, ceftazidime pentahydrate, amoxicillin, ampicillin sodium, cefaclor, cefadroxil, cefamandole sodium, cefazolin sodium, cefoperazone sodium, cefotaxime sodium, cefoxitin sodium, cefsulodin sodium, cephalothin sodium, cephapirin sodium, cephradine, cloxacillin sodium, and penicillin G sodium. The competitors were diluted to afford a solution containing a concentration range of 100−1000 ng/well in column 2 of the 96-well microtiter plates. Each competitor was serially diluted across the plate with columns 11 and 12 serving as controls (i.e. no competitor).

RESULTS

Hapten Synthesis. In these studies the desfuroyl metabolite of ceftiofur was used as the immunogen. Ceftiofur was hydrolyzed to afford desfuroylceftiofur *in situ*, which was then conjugated to either maleimide-activated BSA or maleimide-activated KLH. The presence of the thiol was monitored by TLC in $CHCl_3$−MeOH (1:1), and spots were visualized by development of the TLC in iodine according to the method by Brown and Edwards (1968). The free thiol reacts immediately with the maleimide portion of the heterobifunctional cross-linking reagent sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (*s*-SMCC). KLH-SMCC−desCef was used as the immunizing hapten and BSA-SMCC−desCef used as the plate-coating antigen in the initial ELISA screens. The heterologous plate-coating antigen was prepared using ceftiofur and an amine-specific heterobifunctional cross-linking reagent, *N*-(γ-maleimidobutyryloxy)sulfosuccinimide ester (*s*-GMBS). The free amine on ceftiofur reacts with the succinimide portion of the reagent, and the maleimide portion of the reagent is coupled with sulfhydryl groups on the thiolated carrier protein to yield the hapten−protein conjugate BSA-*s*-GMBS−Cef. All hapten−car-

rier protein conjugates were analyzed by high-resolution nondenaturing gel electrophoresis. The immunogen and the plate-coating antigens gave homogeneous bands that were clearly distinguishable from the carrier proteins BSA, BSA−SMCC, KLH, and KLH−SMCC [data not shown, cf. Rose *et al.* (1995)]. Details of the hapten syntheses can be found in an earlier publication (Rose *et al.*, 1995).

Hybridoma Production. An ELISA was used to screen for anticeftiofur antibody producing hybridoma cells using BSA-SMCC−desCef as the plate-coating antigen. Ten days following cell fusion, 3−5 hybridoma colonies were observed in 90−95% of the wells of each 96-well plate. Positive results (greater than twice that of the background) were observed with the supernatants from 144 wells in the initial screening ELISA. The cells in these were expanded and analyzed by ciELISA for inhibition of antibody binding in the presence of free (unconjugated) ceftiofur on BSA-*s*-GMBS−Cef-coated antigen plates. Only 10 of the original 144 cell cultures were observed to produce antibodies having binding that was inhibitable by ceftiofur. Cells from these 10 hybridomas were subcloned, resulting in the establishment of two stable monoclonal cell lines secreting anticeftiofur antibodies. These cell lines were designated Cef-68 and Cef-116.

MAb Isotyping. The immunoglobulin subclass was determined using an isotype-specific ELISA kit. The MAb produced by hybridoma Cef-68 clones was determined to be an IgG_{2a} antibody with kappa light chains, and the MAb from Cef-116 was established as an IgG_1 antibody with kappa light chains.

Antibody Characterization. The ability of Cef-68 and Cef-116 to recognize free (unconjugated) ceftiofur was evaluated using the ciELISA (Figure 3). The 50% inhibition of control activity (IC_{50}) was observed at 32 ppb for Cef-68. MAb Cef-116 was observed to have an IC_{50} of 0.33 ppb (i.e., 100-fold greater affinity than Cef-68).

Competition ELISA curves were generated for Cef-68 and Cef-116 using a variety of cephalosporins and penicillins (not shown). The data from these experiments are summarized in Table 1. Figure 2 shows the structures for ceftiofur, desfuroylceftiofur, and some of the cephalosporins that were tested for cross-reactivity. MAb Cef-68 was observed to bind with ceftriaxone, cefteram, and cefotaxime. Likewise, MAb Cef-116 bound cefotaxime and ceftriaxone with IC_{50} values comparable to those observed with ceftiofur. Binding to cefteram and cefuroxime was 10- and 100-fold reduced, respectively, as compared to MAb Cef-68. No cross-reactivity was observed with any of the penicillins as shown in Table 1.

Table 1. Competitive Inhibition Values (IC_{50}) for Ceftiofur and Related Compounds

	IC_{50} (ppb) values for antibodies[a]	
competitor	Cef-68	Cef-116
ceftiofur	32.33 ± 0.58	0.33 ± 0.05
ceftriaxone	38.67 ± 8.62	0.33 ± 0.05
cefotaxime	79.67 ± 0.58	0.48 ± 0.05
cefteram	200.00 ± 10.00	3.57 ± 0.75
cefuroxime	−[b]	406.67 ± 130.13
ceftazidime	−	−
cephalothin	−	−
cefoxitin	−	−
cefazolin	−	−
cefadroxil	−	−
cefamandole	−	−
cephradine	−	−
cephapirin	−	−
cefaclor	−	−
cefsulodin	−	−
cefoperazone	−	−
ampicillin	−	−
amoxicillin	−	−
cloxacillin	−	−
penicillin G	−	−

[a] Results are quoted as values ± standard deviation using an average of three to six determinations. [b] −, no competitive inhibition was observed at a competitor concentration of 1000 ng/well.

DISCUSSION

To our knowledge this is the first report of the isolation of monoclonal antibodies that bind ceftiofur sodium. The hapten used to produce these MAbs was designed as a result of previous studies investigating the immunogenicity of different hapten−protein conjugates and plate-coating antigens (Rose *et al.*, 1995). A suitable hapten for immunization should preserve the chemical and physical properties of the target compound; thus, haptens were designed to retain the basic features of the parent ceftiofur molecule. The results from this study are consistent with findings from our earlier studies and suggest that metabolism of the ceftiofur immunogen occurs in the animal. Thus, we used desCef as the immunogen to generate the MAbs. Our results clearly show that linkage through the thiol group of desfuroylceftiofur resulted in MAbs that bound ceftiofur. Linkage via the free amine in ceftiofur did not produce such MAbs (unpublished data). The KLH-SMCC−desCef conjugate elicited a high titer which produced a specific and sensitive antibody for ceftiofur. Detection of anticeftiofur antibodies using BSA-*s*-GMBS−Cef as the plate-coating antigen was the most advantageous. Our results support previous documentation on hapten design criteria as reported in other studies (Goodrow *et al.*, 1990, 1995; Harrison *et al.*, 1989, 1991a,b; Karu *et al.*, 1994; Wie and Hammock, 1984).

Previous attempts in our laboratory to produce monoclonal antibodies directed against ceftiofur sodium have been difficult. This may be due to the nonspecific enzyme-catalyzed cleavage of the thioester bond resulting in the formation of the desfuroylceftiofur and furoic acid metabolites of ceftiofur (Jaglan *et al.*, 1990). Desfuroylceftiofur has been detected in the plasma of calves when treated with labeled ceftiofur (Jaglan *et al.*, 1990), and in other species, such as rats and dogs, it is presumably bound to macromolecules via disulfide bonds (Jaglan *et al.*, 1989).

Although the anti-desCef antibody retained high specificity to ceftiofur, we report that the development of ciELISAs for a variety of ceftiofur analogues was possible using the anti-desCef MAbs Cef-68 and Cef-116. The MAbs were tested for binding of several related cephalosporins and a number of penicillins. The competitors can be grouped into five categories according to their chemical structures: (1) structurally related cephalosporins containing the cephem nucleus, the thiazolyl ring, and the methoxyiminoacetamide oxime (cefotaxime, cefteram, and ceftriaxone); (2) a cephalosporin containing the cephem nucleus, the methoxyiminoacetamide oxime, but a furan ring instead of the thiazolyl ring (cefuroxime); (3) a cephalosporin containing the cephem nucleus, the thiazolyl ring, but a bulky oxime (ceftazidime); (4) cephalosporins containing only the cephem nucleus as a common feature (cefaclor, cefadroxil, cefamandole, cefazolin, cefoperazone, cefox-

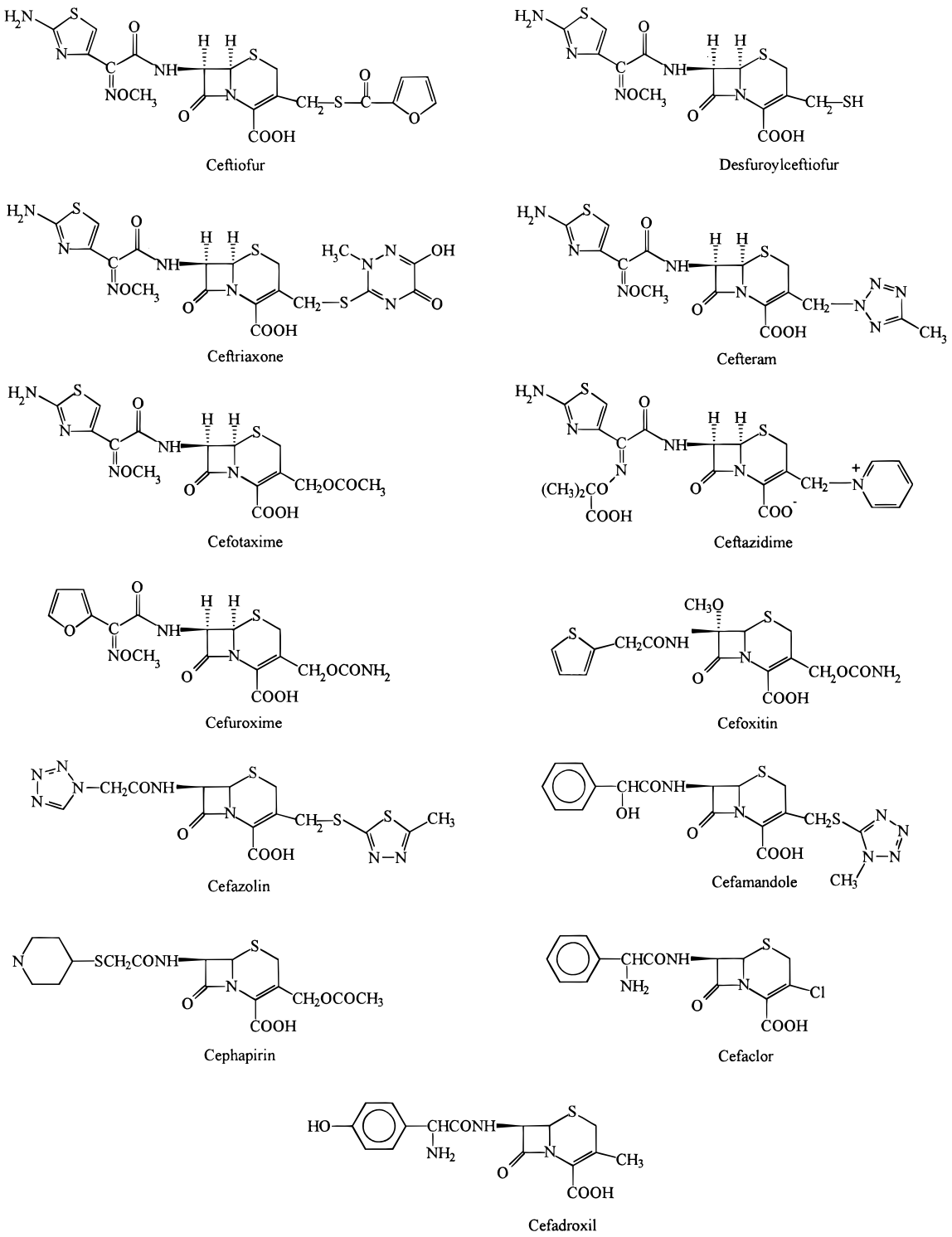

Figure 2. Structures of ceftiofur, desfuroylceftiofur, and a variety of cephalosporins used for cross-reactivity studies.

itin, cefsulodin, cephalothin, cephapirin, and cephradine); and (5) penicillins which have the penem nucleus as a structural feature (amoxicillin, ampicillin, cloxacillin, and penicillin G).

Monoclonal anti-desCef antibodies from both Cef-68 and Cef-116 recognized all cephalosporins in category 1. However, Cef-68 has a lower relative affinity for these compounds than does Cef-116. In contrast, binding to the category 2 cephalosporin, cefuroxime, was minimal due to the absence of the thiazolyl ring. MAbs Cef-68 and Cef-116 are different in that Cef-68 could not detect cefuroxime at all. This suggests that the thiazolyl ring is an important structural feature in the antibody−antigen binding process. Even though the thiazolyl ring fragment is present in ceftazidime (category 3), this compound could not be bound, most likely due to the presence of the bulky oxime, which presumably inhibits binding by the introduction of steric congestion into the binding site. The fact that the MAbs did not recognize ceftazidime suggests that the methoxyiminoacetamide fragment is also an important structural feature necessary for antibody binding. None of the category 4 cephalosporins, containing only the cephem nucleus and no common functional groups, and none of the category 5 penicillins were detected by the MAbs. The fact that the antibodies do not "recognize"

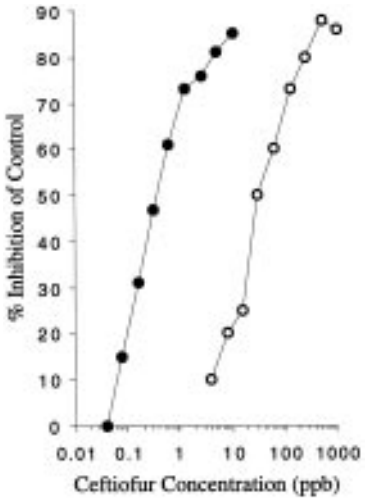

Figure 3. Typical cELISA curves with monoclonal antibodies Cef-68 (open circles) and Cef-116 (solid circles) using ceftiofur as competitor.

the cephem nucleus alone suggests that neither the general cephems nor penems contain important structural and electronic features necessary for antibody binding in this case.

Our cross-reactivity study using the ciELISA shows that certain components of the molecule (epitopes) must be present for antibody binding to occur. Determining the contribution that each part of the ceftiofur molecule makes to antibody binding is important for understanding the phenomenon of cross-reactivity between related cephalosporins. The combining sites of the MAbs reported here require the thiazolyl and methoxyiminoacetamide fragments; thus, the antibodies are recognizing the entire 2-(2-aminothiazol-4-yl)-2-methoxyiminoacetamide fragment.

Even though an antibody has a unique three-dimensional structure, it may be multispecific in that it is able to combine either with the immunizing hapten or with antigens of similar or disparate structures (Richards *et al*., 1975). The degree of antibody specificity is determined by the extent of complementarity between the hapten and the amino acid sequence present in the antibody combining site. Thus, the cross-reactivity observed here arises due to the similarity of epitopes between ceftiofur, ceftriaxone, cefteram, and cefotaxime.

CONCLUSION

It has been reported that varying the cross-linker, hapten, and the site of attachment to produce a heterologous assay system can result in a more sensitive ELISA (Van Weemen and Schuurs, 1975). We have reported a highly sensitive and accurate ELISA for ceftiofur and four of its analogues using a heterologous assay system. The MAbs reported here show limited cross-reactivity and are specific for ceftiofur and only those cephalosporins that have structural and electronic features that resemble those of ceftiofur closely. The ciELISA developed here is useful for the analysis of ceftiofur in other media. Preliminary studies of the ciELISA format in milk suggest that we can detect ceftiofur at a few parts per million without any cleanup. We have developed a new method for the detection of ceftiofur, which is fast and easy to perform and does not require expensive and sophisticated equipment.

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