Suppression of Lymphocyte Responses by Cephalosporins

E. A. CHAPERON* AND W. E. SANDERS, JR.

Department of Medical Microbiology, Creighton University, Omaha, Nebraska 68178

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Human peripheral blood lymphocytes were cultured in several concentrations of each of several cephalosporins. Responses to phytohemagglutinin were compared with that of duplicate cultures containing penicillin-streptomycin, chloramphenicol, or no antibiotics. Possible effects of cephalosporins on responses of lymphocytes to concanavalin A and pokeweed mitogen were similarly determined. Significant suppression of responses to phytohemagglutinin and concanavalin A were seen in cultures containing 50 μg each of cephalothin, cephalxin, or cephradine per ml. Lymphocyte responses to pokeweed mitogen were suppressed by 50 μg of cephalxin, cephradine, or cefoxitin per ml. A higher concentration (100 μg/ml) of all cephalosporins except cefoxitin and cefazolin suppressed the phytohemagglutinin response to less than 20% that of controls. Chloramphenicol (50 μg/ml) did not inhibit the response to any mitogen used. These findings suggest that cephalosporins should not be used for prevention of bacterial overgrowth in certain cell cultures. Since many of the cephalosporins were suppressive in therapeutically attainable concentrations, these results may have potential clinical significance.

The cephalosporins are a group of semisynthetic antibacterial agents that are chemically related to the penicillins (18). The nucleus of the cephalosporin molecule, 7-aminocephalosporanic acid, differs from that of the penicillins only in that the beta-lactam ring of the former is fused to a six-membered heterocyclic ring rather than a five-membered ring. The side chains at positions 3 and 7 of cephalosporanic acid are the major variables in the structures of the different cephalosporins. Their mode of action is essentially the same as that of the penicillins.

In spite of their structural resemblance to the penicillins, most, but not all, patients with a history of penicillin allergy can be treated safely with the cephalosporins (14). Nevertheless, a number of adverse reactions have been reported. Rashes, urticaria, eosinophilia, and rare cases of neutropenia have been associated with the administration of these drugs to patients with or without a past history of penicillin allergy (1, 7, 11, 14). Other side effects associated with the use of cephalosporins include the frequent occurrence of a positive Coombs test in patients receiving high doses of cephalothin (12, 15). Also, Sanders et al. (13) have reported the development of a serum sickness-like syndrome in each of 30 individuals who received high doses of cephalothin or cepahpirin for 2 to 4 weeks.

A proposed mechanism that seems to explain the positive Coombs reaction seen in cephalosporin-treated patients implicates the binding of the drug, or drug-protein complexes, to erythrocyte membranes (6, 7, 12). This phenomenon might also relate to the serum sickness-like syndrome. An alternative hypothesis could be proposed in which some effect by the drugs on thymus-dependent (T) lymphocytes might be implicated. There is evidence from animal studies that the thymus and T lymphocytes serve a regulatory role, not only dictating the antigens to which the immune system will respond (9), but also controlling the magnitude of the response (2) and perhaps even determining the classes of immunoglobulins to be synthesized (2, 3). There has been considerable interest in recent years in the possible role of T-lymphocyte dysfunction in a variety of allergic and autoimmune diseases (10, 16). The present study was initiated to screen cephalosporins for possible adverse effects on lymphocyte responses to stimulation with the plant mitogens phytohemagglutinin (PHA), concanavalin A (ConA), and pokeweed mitogen (PWM).

MATERIALS AND METHODS

Antimicrobial agents. The cephalosporins were obtained in powdered form from the manufacturers. Cefamandole (lot 81-88-5A), cephalothin (NDC 0002-7001-01), cephalaxin (QA 153D, lot 454--226-20), and cefazolin (QA 184N, lot 8K229) were provided by Eli Lilly and Co., Indianapolis, Ind. Cefoxitin (lot L-620, 338-01B22) was obtained from Merck Sharp & Dohme.
SUPPRESSION OF LYMPHOCYTE RESPONSES

West Point, Pa.; cepahpin (lot 73F1697) was from Bristol Laboratories, Syracuse, N.Y.; and cephradin (D-39304, lot SJB-6732-265-A) was from Smith Kline & French Laboratories, Philadelphia, Pa. The 7-aminopenicillosporanic acid was obtained from Aldrich Biochemicals, Milwaukee, Wis., and chloramphenicol (lot H717053) was purchased from Parke, Davis and Co., Detroit, Mich. Penicillin-streptomycin preparations were obtained from Grand Island Biological Co., Grand Island, N.Y.

Lymphocyte separation. Venous blood from presumably healthy donors was mixed with an equal volume of physiological saline, containing 20 U of sodium heparin (Eli Lilly & Co., Indianapolis, Ind.) per ml. Portions of the diluted blood were layered over Ficoll-Hypaque and centrifuged by the method of Thorsby and Bratlid (17). The isolated lymphocytes were washed, first in physiological saline and then in Eagle minimal essential medium containing 10% fetal calf serum. After the second washing, the cells were suspended in fresh minimal essential medium and counted under phase optics, trypsin blue exclusion being used as an indicator of cell viability, and the suspension was diluted to 5 x 10⁶ cells per ml in culture medium.

Lymphocyte cultures. Portions (1 ml each) of the lymphocyte suspensions were distributed into plastic culture tubes (Falcon Plastics, Oxnard, Calif.) and incubated at 37°C in 5% CO₂ for 96 h. PHA-P (8.5 μg/ml; Difco Laboratories, Detroit, Mich.), ConA (3.0 μg/ml; Sigma Chemical Co., St. Louis, Mo.), or PWM (100 μg/ml; Difco) were used as the mitogen. The antimicrobial agents were diluted in 0.005 M barbital buffer. Both mitogens and antimicrobial agents were added to the cultures at the beginning of incubation. At 72 h, 0.5 μCi of [methyl-³H]thymidine (specific activity, 2.0 μCi/mmole; New England Nuclear Corp., Boston, Mass.) was added to each culture.

Liquid scintillation counting. The cultures were killed at 96 h by the addition of 1.0 ml of cold 10% trichloroacetic acid. The precipitates were washed three times with 5% trichloroacetic acid, solubilized by the addition of 0.5 ml of 0.3 N NaOH, and incubated for 20 min at 37 to 45°C. Next, 0.5 ml of warm 20% trichloroacetic acid was added, and the loosely capped tubes were incubated at 88 to 90°C for 20 min. After cooling and centrifugation, 0.5 ml of supernatant from each tube was added to 10 ml of Aquasol (New England Nuclear Corp.) and counted in a Packard liquid scintillation counter. After subtraction of background counts per minute, responses in cultures containing cephalosporins and other antibiotics were expressed as percentages of responses in similar cultures containing no antibiotics.

T-cell (E) rosettes. Washed peripheral blood lymphocytes obtained from Ficoll-Hypaque gradients were diluted to 6.0 x 10⁶ cells per ml in minimal essential medium. To 0.2-ml portions of this suspension, 0.1 ml of cephalosporin (50 μg) or penicillin-streptomycin (50 U:50 μg) was added. After incubation for 30 min at 37°C, 0.3 ml of 0.5% washed sheep erythrocytes was added to each tube. The tubes were centrifuged at 150 x g for 5 min and incubated at 4°C for 1 h. The cells were then gently suspended, and 200 cells were scored for rosette formation. A rosette was identified as a lymphocyte with three or more sheep erythrocytes attached to it.

RESULTS

Lymphocyte responses to PHA. The effect of adding different cephalosporin concentrations to the culture medium on the lymphocyte response to PHA is indicated in Fig. 1. Each point represents the mean of three replicate cultures, and all points on a given curve were obtained from a single experiment. Significant suppression of responses to this mitogen was seen in the cultures containing 50 μg of cephalothin (32% of controls), cephradin (24%), or cephalaxin (15%) per ml. Cultures containing 100 μg of any one of these three drugs per ml were suppressed to 5% or less of controls. Responses in cultures containing 100 μg of cephamandole, cepahirin, or 7-aminopenicillosporanic acid per ml were reduced to less than 20% that of controls. Except for cefazolin, 200 μg of all drugs tested per ml reduced the PHA response to less than 16% that of controls. This level of cefazolin reduced the response to 44%. It is noteworthy that the greatest suppression was seen on the cultures containing cephalaxin or cephradin, two compounds that are structurally very similar, since they are both methylated at the 3 position and differ from each other only in the number of double bonds in the six-membered ring at the 7 position.

When responses to PHA, in the presence of 50 μg of each of the cephalosporins per ml, were compared in a single experiment, the results shown in Fig. 2 were obtained. Responses to PHA were reduced to less than 20% that of controls in cultures containing cephalaxin or cephradin and to approximately 50% in those containing cephalothin. All other cultures, including those containing 50 μg of chloramphenicol per ml, responded as well as control cultures containing no antibiotics.

Lymphocyte responses to ConA. The effect of cephalosporins on lymphocyte responses to stimulation with ConA is shown in Fig. 2. The inhibitory effects of cephalaxin and cephradin were even more striking here than they were in the PHA experiments. Also, the response to ConA in cultures containing 50 μg of cephalothin per ml was reduced to approximately 10% that of controls. A reduced response to ConA in control cultures containing only the barbital buffer (80 μg/ml) used to dilute the antimicrobial agents was seen in several experiments. Since the addition of this buffer did not inhibit the responses to PHA and PWM and since the ConA response was also inhibited by cephalosporins diluted in culture medium, we concluded...
that the effect was probably due to an interaction between barbital and ConA.

The effects of different concentrations of cephalexin, cefradine, and cephalothin on the responses to ConA in separate experiments are illustrated in Fig. 3. Again, at all concentrations of these drugs, the greatest suppression was seen in cultures containing cephalexin or cefradine.

**Lymphocyte responses to PWM.** Responses to PWM, in the presence of 50 μg of the different cephalosporins per ml, are shown in Fig. 2. Results here were more variable than those seen with PHA or ConA but were consistent in that responses in the presence of cephalexin and cefradine were greatly depressed. Also, cefoxitin inhibited the lymphocyte response to this mitogen.

PWM responses in the presence of different concentrations of cefradine, cephalexin, cefoxitin, and penicillin-streptomycin are shown in Fig. 4. Penicillin-streptomycin concentrations were 25 U:25 μg, 50 U:50 μg, 100 U:100 μg, and 200 U:200 μg. The data clearly indicate that for these four different concentrations of penicillin and streptomycin, with triplicate determinations at each concentration, there was no suppression...
of the response to PWM. The suppression of the responses to PWM in the presence of the cephalosporins, however, was consistent with the earlier data.

Mechanism of action. The possibility that the cephalosporins might be inhibiting mitogenesis by binding to, or otherwise altering, cell membranes was explored by determining the possible effect of preincubating lymphocytes (for 30 min in the presence of 50 μg of drug) on their ability to form E rosettes with sheep erythrocytes. The data (Table 1) indicate that, of the drugs tested, cefoxitin and cephalothin both reduced the number of rosettes, whereas cephradine, cephalaxin, and cefazolin had no effect. In a second experiment designed to answer the same question, it was found that preincubation of lymphocytes for 1 h at 37°C, in the presence of 50 μg of cephalothin, cephradine, cephalaxin, or cefoxitin per ml, did not inhibit the mitogenic response when the cells were subsequently washed and cultured in antibiotic-free medium. When cephalosporins were added 24 h after the initiation of lymphocyte cultures, a similar but decreased level of suppression in the responses to all three mitogens was observed (Table 2).

DISCUSSION

This study clearly demonstrates that cephalosporins can suppress in vitro lymphocyte responses to the mitogens PHA, ConA, and PWM. That this effect may be due to direct killing seems unlikely, since cell viability studies, using the trypan blue exclusion method, revealed that the drugs did not affect lymphocyte viability. Also, observed differences in the degree of suppression by different cephalosporins cannot be related to differences in the stability of the drugs in cell culture, since cephalothin, a relatively unstable compound, and cephradine, one of the more stable cephalosporins (18), were both among the more suppressive agents.

Studies of lymphocyte responses to mitogenic substances have revealed several cellular requirements for cell activation (8), and interference with any of these is sufficient to reduce the blastogenic response. Different plant mitogens react specifically with different glycopep-
tide receptors on the lymphocyte, and blastogenic responses can be suppressed by agents that interfere with the binding of stimulants to these receptors. Serum glycopeptides can act as competitive inhibitors for mitogen binding, and at least one antimicrobial agent, polymyxin B, is known to inhibit lymphocyte responses to lipopolysaccharide by binding to the mitogen (4).

Cephalixin and cephradine both markedly suppress lymphocyte responses to all three mitogens used in this study, suggesting that these cephalosporins are probably acting by some mechanism other than a specific interference with mitogen binding to the cell membrane. We have also found that preincubation of lymphocytes in the presence of cephalothin, cephradine, or cephalixin did not inhibit the mitogenic response when the cells were subsequently washed and cultured in antibiotic-free medium. This would indicate that the drugs are not binding to mitogen-specific receptors on the lymphocyte membrane.

Activation of lymphocytes by both specific and nonspecific mitogens requires interaction between different cells, either direct interaction between lymphocytes or interactions involving macrophages, and also perhaps erythrocytes. Interference with cell aggregation by shaking the cultures or addition of substances such as hyaluronic acid, which prevents cell aggregation, will inhibit the response (8). The possibility exists

**TABLE 1. Effect of 50 μg of cephalosporins on E-rosette formation by human lymphocytes**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Rosette formation (%) *</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>56</td>
</tr>
<tr>
<td>Penicillin-streptomycin (50 U:50 μg)</td>
<td>67</td>
</tr>
<tr>
<td>Cephradine</td>
<td>57</td>
</tr>
<tr>
<td>Cephalixin</td>
<td>52</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>55</td>
</tr>
<tr>
<td>Cefotaxin</td>
<td>39</td>
</tr>
<tr>
<td>Cefalothin</td>
<td>37</td>
</tr>
</tbody>
</table>

*Percentage of 200 lymphocytes counted that formed E rosettes.**
that the cephalosporins in the culture medium might interfere with cell aggregation, although morphological changes, which might be attributed to the presence of drugs, were not noted. Preliminary data indicate that cefoxitin and cephalothin both inhibit the binding of sheep erythrocytes to human T lymphocytes (E-rosette formation). Other inhibitory cephalosporins, however, do not interfere with E-rosette formation (cf. Table 1).

There are many biochemical events between the initial interaction of the lymphocyte with the mitogen and the start of deoxyribonucleic acid synthesis, and it is difficult to identify a single mode of action for inhibitors. Since drugs that act at this level are usually toxic for any metabolically active tissue, e.g., bone marrow, their effects would be readily apparent. It would seem unlikely that the cephalosporin effects seen in the present study are due to such a mechanism. However, this possibility cannot be excluded since a decreased level of suppression occurs when cephalosporins are added 24 h after the initiation of lymphocyte cultures.

Although one should be extremely cautious when attempting to attach any physiological significance to in vitro phenomena, it is tempting to speculate. Of particular interest is the possibility that, in patients manifesting allergic reactions to cephalosporins, the drugs may be acting selectively on T lymphocytes as potentiators of antibody production, particularly immunoglobulin E, against antigens of otherwise low immunogenicity, including self-antigens. There is evidence from animal studies to support such a hypothesis (2, 3, 5), and there have been reports that atopy in humans may be associated with a defect in a subpopulation of T cells (6, 16). It should be noted that the levels of cephalosporins used in this study are therapeutically attainable with many of these drugs. One approach to this problem might be an attempt at inducing the production of reaginic antibodies in mice to a protein antigen, e.g., ovalbumin, by maintaining high blood levels of cephalosporins. Also, the possible effects of cephalosporins on specific immune responses known to be mediated by T cells need to be explored.

These studies serve to call attention to the sensitivity of lymphocyte mitogenic responses to additives in the culture medium. Although several of the cephalosporins caused severe depression of the lymphocyte responses to mitogens, other antimicrobial agents such as penicillin, streptomycin (and other aminoglycosides), and even chloramphenicol were nonsuppressive (cf. 8). This should be kept in mind when selecting antimicrobial agents for use in cell cultures. The possible relationship of the suppression of lymphocyte responses by cephalosporins to the high rate of serum sickness-like reactions in patients given high doses for prolonged periods merits further study.

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LITERATURE CITED