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Chlorphenesin: an Antigen-Associated Immunosuppressant

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Chlorphenesin (3-*p*-chlorophenoxy-1,2-propanediol), when injected intravenously together with either of two common bacterial antigens, inhibits the antibody response of the rabbit. The antigens studied are those common to *Enterobacteriaceae* and to gram-positive bacteria. The immunosuppression is contingent upon incubation of chlorphenesin and antigen in vitro prior to administration, since separate injection of antigen and inhibitor or of mixtures without prior incubation yields undiminished antibody response. Chlorphenesin, as shown by hemagglutination-inhibition tests, does not alter the antigenic determinants, because antibody neutralization occurs in the presence or absence of the drug. The immunosuppressive effect is reversible, since precipitation of chlorphenesin at 4 C substantially restores immunogenicity. Animals immunized with antigen-drug mixtures, which fail to respond with significant antibody production, nonetheless are immunologically primed. It is concluded that chlorphenesin represents another example of antigen-associated immunosuppressants.

Previous studies from this laboratory have shown that various substances, such as lipopolysaccharide and its lipoid A fraction, cardiolipin, serum, and alpha-one plasma protein, suppress the antibody response of the rabbit to certain bacterial antigens, provided that the inhibitor is mixed with the antigen in vitro prior to immunization (6, 10, 12-16). Such inhibitors have been referred to as antigen-associated immunosuppressants (16). Recently, Berger et al. (1) reported a similar immunosuppressive phenomenon induced by chlorophenesin (3-*p*-chlorophenoxy-1,2-propanediol) with erythrocytes as antigen. The present investigation was carried out to determine the effects of chlorophenesin on the immune response of rabbits to two bacterial antigens, one shared by gram-positive bacteria and first described by Rantz et al. (7) and the other shared by *Enterobacteriaceae* and discovered by Kunin et al. (4).

MATERIALS AND METHODS

The common antigen of gram-positive bacteria was prepared as follows. Strains of *Staphylococcus aureus* (coagulase-positive) and *Bacillus subtilis* were grown on brain-veal-agar in Kolle flasks at 37 C for 18 hr. The microorganisms were suspended in 25 ml of phosphate hemagglutination buffer (*p*H 7.3; Difco) per flask. The supernatant fluid obtained after centrifugation of the bacterial suspension at 23,500 × *g* for 20 min was used as antigen. In addition, the ethanol-insoluble fraction, prepared by the previously described procedure (8, 9), was utilized. The common enterobacterial antigen (CA−) was obtained by ethanol fractionation from *Salmonella typhimurium* and *Escherichia coli* O111 and O14. Immunogenic CA of the former two microorganisms is ethanol-soluble and that of *E. coli* O14 is insoluble (8, 9). Chlorphenesin (3-*p*-chlorophenoxy-1,2-propanediol), obtained through the courtesy of G. M. Fukui and F. M Berger, Wallace Laboratories, Cranbury, N. J., was dissolved to a concentration of 10 mg/ml in phosphate hemagglutination buffer (*p*H 7.3; Difco) and heated at 56 C in a water bath for 30 min. This solution was kept at 37 C until used.

Groups of three to four albino rabbits, weighing approximately 2 to 3 kg, were immunized by three intravenous injections of 1 ml each of antigen alone or of antigen and chlorophenesin, the interval between injections being 3 to 4 days. The amounts of antigen and chlorphenesin used are given below.

The antibody response was measured by means of the previously described passive hemagglutination test (10, 15, 16) on serum samples obtained before, during, and after immunization. Briefly, rabbit erythrocytes (2.5%) were washed, and the sediment was then resuspended to a concentration of 2.5% in indicator antigen, either supernatant fluid of *B. subtilis* (1:10) for titration of CA(+) antibodies or ethanol-soluble CA(−) of *E. coli* O111 (1:10) for titration of CA(−) antibodies. The mixtures were incubated in a water bath at 37 C for 30 min. Serum in serial twofold dilutions (0.2 ml)

TABLE 1. *Effect of chlorphenesin on hemagglutinin response of rabbits to common antigen of gram-postive bacteria (CA+)*

Group	Materials for immunization	Mean CA(+) hemaglutinin titers[a] (reciprocal)			
		Day 0	Day 7	Day 10	Day 14
1	Mixture of CA(+) and buffer	<10	120	340	560
2	Incubated mixtures of CA(+) and				
	chlorophenesin (10 mg/ml)	<10	<10	<10	<10
	chlorphenesin (1 mg/ml)	<10	20	20	30
	chlorphenesin (0.1 mg/ml)	<10	80	373	327
	chlorphenesin (0.01 mg/ml)	<10	107	533	747
3	Nonincubated mixture of CA(+) and chlorphenesin (10 mg/ml)	<10	80	267	320
4	CA(+) and chlorphenesin (10 mg/ml) injected separately	<10	100	320	640

[a] Day 0, preimmunization; days 7, 10, and 14, postimmunization.

was mixed with equal amounts of antigenically modified erythrocytes (2.5%). The mixtures were incubated at 37 C for 30 min. The resulting hemagglutination was read after centrifugation at 1,300 × *g* for 2 min. The specificity of the antibody was documented by hemagglutination-inhibition tests, as described previously in detail (8, 17). Throughout the experiments, phosphate hemagglutination buffer was used as diluent.

RESULTS

In the first series of experiments, the effect of chlorphenesin on the hemagglutinin response to the common antigen of gram-positive bacteria (CA+) was investigated.

Groups of three to four rabbits were immunized by three intravenous injections on days 0, 3, and 7, with 1 ml of antigen alone or of antigen and chlorphenesin. (i) The first group received a mixture of one part of CA(+) antigen (final dilution of 1:100) and nine parts of buffer and served as the control. (ii) The second group received a mixture of one part of antigen and nine parts of chlorphenesin in concentrations of 10, 1, 0.1, or 0.01 mg/ml, respectively. Before injection, these mixtures were incubated at 37 C for 30 min. (iii) The third group received the same antigen-chlorphenesin mixtures without prior incubation. (iv) The fourth group received antigen and chlorphenesin by simultaneous, albeit separate, injections. The results of a representative experiment are shown in Table 1. Chlorphenesin markedly inhibited the CA(+) hemagglutinin response when used in amounts of 1 or 10 mg/ml but not in amounts of 0.1 or 0.01 mg/ml. It can also be noted that injection of the nonincubated mixture as well as separate administration of the two materials into different ear veins resulted in an undiminished immune response. Similar results were obtained with the ethanol-insoluble fraction of supernatant fluid of the staphylococcal culture. In one such experiment, chlorphenesin in amounts of 10 mg/ml suppressed antibody production completely (reciprocal titer, < 10) and in amounts of 1 mg/ml suppressed it substantially (titer, 20); the reciprocal titer of control animals immunized with antigen alone was 320.

Further experiments revealed that chlorphenesin suppresses antibody formation less effectively when larger amounts of antigen are used. Conversely, with smaller amounts of antigen, the drug partially inhibits the antibody response even in a concentration of 0.1 mg/ml. That the immunosuppressive effect results not merely in a delay of antibody production is evident from the finding that antibody titers did not rise during an observation period of 4 weeks after initiation of immunization.

Additional studies have revealed that rabbits immunized with an incubated mixture of antigen and chlorphenesin are immunologically primed, as shown by the results of the following experiment. The first group of rabbits received three intravenous injections, 3 to 4 days apart, of a mixture of CA+ (1:100) and phosphate buffer and served as a control. The second group was immunized in the same manner with an incubated mixture of CA+ (1:100) and chlorphenesin (10 mg/ml) but, on day 14, was boosted with a single injection of a subeffective dose (1 ml) of CA+ (1:100). The third group of nonprimed rabbits received only the booster dose of antigen. The results are shown in Fig 1. Immunization with the mixture of chlorphenesin and antigen results in priming, since the antibody titers increased after booster injection to significantly higher levels than those found in nonprimed control animals. The immunosuppressive effect of the drug is evident from the results on animals injected with antigen alone.

The above results of the immunosuppressive effect of chlorphenesin could be readily explained if the antigenic determinant were to be inactivated or blocked. The following lines of evidence indi-

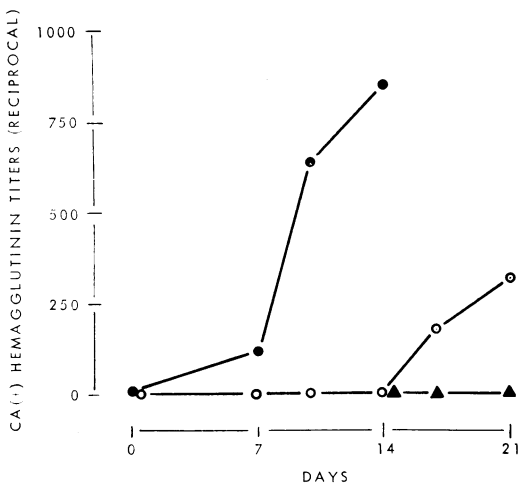

FIG. 1. *Effect of chlorphenesin on immunological priming by common antigen of gram-positive bacteria. Symbols:* ●, *rabbits immunized with antigen alone;* ○, *rabbits immunized with an incubated mixture of antigen and chlorphenesin and boosted with antigen;* ▲, *animals injected with booster dose of antigen only.*

cate that this hypothesis is not tenable. Firstly, it was shown that rabbits immunized with the antigen-chlorphenesin mixture are immunologically primed. Secondly, hemagglutination-inhibition tests revealed that the antigen is capable of reacting with the antibody even in the presence of the drug. Since chlorphenesin in concentrations higher than 1 mg/ml is hemolytic (3), serial dilutions of the antigen-chlorphenesin mixture, prepared in the same ratio (antigen, 1:10, and chlorphenesin, 1 mg/ml) as employed for immunization, were used. It was found that the minimal inhibitory amount of antigen was the same (1:100), both in the presence and in the absence of the drug. Thirdly, advantage was taken of the fact that soluble chlorphenesin precipitates at 4 C. To this end, an incubated mixture of CA and chlorphenesin was prepared and cooled to 4 C; the supernatant fluid obtained therefrom was used for immunization together with appropriate controls. The results of this experiment are recorded in Table 2 and indicate that immunogenicity of the antigen was substantially restored after precipitation of the drug. Taken together, these observations suggest that chlorphenesin does not irreversibly alter the antigenic determinant and probably does not block it.

The effect of chlorphenesin on the common enterobacterial antigen was explored next. The results of a representative experiment are summarized in Table 3. It can be seen that the incubated mixture of antigen and chlorphenesin is substantially less immunogenic than the antigen alone, that incubation of antigen and drug is required to obtain immunosuppressive effects, and that separate injection of the two materials does not result in significant immunosuppression. Thus, chlorphenesin inhibits antibody production to this antigen under essentially identical conditions as are required with the common antigen of gram-positive bacteria. Since immunogenic CA produced by *E. coli* O14 is

TABLE 2. *Reversibility of immunosuppressive effect of chlorphenesin to common antigen of gram-positive bacteria (CA+)*

Group	Materials for immunization	Mean CA(+) hemagglutinin titers[a] (reciprocal)			
		Day 0	Day 7	Day 10	Day 14
1	Incubated mixture of CA(+) and buffer	<10	120	640	853
2	Incubated mixture of CA(+) and chlorphenesin (10 mg/ml)	<10	<10	<10	<10
3	Supernatant of mixture of CA(+) and chlorphenesin (10 mg/ml) after cooling to 4 C	<10	100	267	320

[a] Day 0, preimmunization; days 7, 10, and 14, postimmunization.

TABLE 3. *Effect of chlorphenesin on hemagglutinin response of rabbits to common enterobacterial antigen*

Group	Materials for immunization	Mean CA(−) hemagglutinin titers[a] (reciprocal)			
		Day 0	Day 7	Day 10	Day 14
1	Mixture of CA(−) and buffer	<10	80	267	320
2	Incubated mixture of CA(−) and chlorphenesin (10 mg/ml)	<10	17	20	20
3	Nonincubated mixture of CA(−) and chlorphenesin (10 mg/ml)	<10	67	213	213
4	CA(−) and chlorphenesin (10 mg/ml) injected separately	<10	80	213	267

[a] Day 0, preimmunization; days 7, 10, and 14, postimmunization.

ethanol-insoluble, additional experiments were carried out with this fraction. It was observed that chlorphenesin in amounts of 10 mg/ml reduced the antibody response by more than 90% under the above conditions.

The question presents itself of whether chlorphenesin acts as an immunosuppressant of the antibody response of the rabbit to other antigens as well. Limited experiments with purified lipopolysaccharide of *E. coli* O113 revealed that immunosuppression was not observed when antigen (total dose per rabbit, 3.7 μg) was incubated with the drug (total dose, 22 mg) prior to immunization. In one such experiment, the maximal mean antibody titers were 1:640 for rabbits receiving mixtures of antigen and chlorphenesin and 1:747 for animals injected with antigen alone. Even when relatively larger amounts of drug were used (total amounts of lipopolysaccharide per rabbit being 1.35 μg and of chlorphenesin 60 mg), inhibition of antibody response did not occur. In addition, chlorphenesin (10 mg/ml) did not inhibit antibody production against O antigen when supernatant fluid of agar-grown cultures of *E. coli* O111 was used for immunization.

DISCUSSION

Inhibition of the antibody response can be effected by specific and nonspecific means. Administration of antigen may result in immunological unresponsiveness, and passively administered antibodies may inhibit formation of antibodies, presumably by a feedback mechanism. Nonspecifically, the immune response can be altered by varied agents, including X irradiation, corticoid hormones, and immunosuppressant drugs. A third type of immunosuppression has come to light, in which the immunosuppressant must be administered together with the antigen. Materials which are effective in this latter system are endotoxic lipopolysaccharides and their lipid A components, cardiolipin, and alpha-one serum protein (6, 10, 12–16). Thus far, this type of immunosuppression has been established only with certain bacterial antigens, namely, the common antigens shared by either *Enterobacteriaceae* or gram-positive bacteria. The present investigation has revealed that chlorphenesin should be added to this list. In this study, it has been shown that chlorphenesin in milligram amounts inhibits antibody production of the rabbit, provided that antigen and drugs are preincubated prior to immunization but not when the two materials are injected separately, albeit simultaneously, or together without incubation. Evidence has been obtained suggesting that chlorphenesin does not act by destruction or blockage of the antigenic determinants. Previously, Berger et al. (1) observed that chlorphenesin, when administered together with sheep or chicken erythrocytes or with penicillin conjugates, suppresses the antibody producing capacity of spleen cells as well as the formation of humoral antibodies. Fukui et al. (3) provided evidence that the drug does not affect the antigen, destroy complement, or interfere with the reaction between complement and antigen-antibody complex. In addition, this drug suppresses hypersensitivity reaction to penicillin in guinea pigs (2) and inhibits IgE-mediated histamine release from human leukocytes (5). Whether a single mechanism is responsible for these varied effects of the drug is unknown. As far as the common enterobacterial antigen is concerned, it has been established that aggregated, but not soluble, antigen is immunogenic (11). The question, therefore, arises of whether chlorphenesin affects aggregation. In view of the fact that the drug inhibits the antibody response to erythrocytes, it is unlikely that this explanation is tenable. Since chlorphenesin exerts immunosuppressive effects only when given together with antigen, either erythrocytes or certain bacterial antigens, it may be postulated that drug and antigen must reach the identical cells and that chlorphenesin affects the uptake or intracellular processing of antigen required for the immune response, or both, resulting in the production of humoral antibodies.

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