Effect of Chlorphenesin on Localized Hemolysis in Gel Assay

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Chlorphenesin, a simple glycerol ether, when added to Jerne plates greatly reduced the number of hemolytic plaques. This effect appeared to be related to dose, and was clearly demonstrable with antibody-forming spleen cells from mice that had been immunized either with sheep red blood cells or with penicillin G conjugated with Keyhole limpet hemocyanin. Chlorphenesin did not affect the antigen, destroy complement, or interfere with the interaction of complement and the antigen-antibody complexes. Incubation of spleen cell suspensions with chlorphenesin prior to plating was more effective in reducing the number of plaques than was addition of the substance to the plates. It may act by reducing the ability of antibodies to react with antigens or by affecting the release of antibodies from the spleen cells.

It has been shown that chlorphenesin (3-*p*-chlorphenoxypropanediol) and certain related phenoxypropanediols can suppress passive cutaneous anaphylaxis elicited in guinea pigs with penicillin conjugates (4, 5). In order to study the mechanism for this suppression, experiments were performed utilizing an adaptation of the localized hemolysis in gel (LHG) technique in which penicillin (KPG) conjugated to protein carriers was injected into mice. It was noted that chlorphenesin added to the agar or to the spleen cell suspension prior to plating reduced the number of hemolytic plaques (G. S. Chandlee et al., *Bacteriol. Proc.*, p. 66, 1968). However, it soon became apparent that chlorphenesin also exerted similar effects on LHG responses in which penicillin did not play a part. Thus, chlorphenesin also reduced the number of hemolytic plaques when added to Jerne plates that contained spleen cells from mice that had been immunized with sheep red blood cells (S-RBC). The experiments carried out in order to understand these observations are the subject of this paper.

MATERIALS AND METHODS

Immunization. Random-bred male Swiss-Webster mice that weighed approximately 20 g were obtained from Carworth Farms, Inc., New City, N.Y., or from Charles River Mouse Farm, Inc., North Wilmington, Mass. About 10^6 S-RBC that had been washed three times with physiological saline were injected intravenously in a volume of 0.1 ml. The KPG conjugates were injected into the tail vein in a volume of 0.1 ml.

Penicillin G conjugates. Penicillin G-Keyhole limpet

hemocyanin (KLH) conjugates for immunization were prepared by the method of Levine (11), and the LHG assay was carried out by a modification of the method described by Massie and Frick (13). KPG was converted to the benzyl penicilloyl derivative (BPO) prior to conjugation. KLH (2 mg) was dissolved in 1.0 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer. To this solution, 8 mg of KPG was added. The pH was adjusted to 11.0 with 1 N NaOH and maintained at room temperature for 2 hr. The pH was then adjusted to 7.0 to 7.2 with 1 N HCl, and the mixture was dialyzed in 0.02 M K_2HPO_4 for 3 days at 4 C with daily changes of fresh K_2HPO_4 solution. Penicillin G conjugated in a similar manner with bovine hemoglobin, bovine serum albumin, bovine gamma globulin, human gamma globulin, or ovalbumin did not induce adequate immune responses detectable by the Jerne technique.

LHG assay. Three or four days after immunization, the mice were killed. Their spleens were extirpated and weighed, and the spleen cells were teased out, suspended in 1.0 ml of Eagle's medium fortified with glutamine, and maintained at 0 C in an ice bath. The plate assay for hemolytic plaques was carried out according to the method described by Braun and Nakano (6), which was based essentially on the techniques of Jerne (9) and Ingraham and Bussard (8).

The detection of penicillin antibodies by the LHG technique was carried out in the following manner. The washed and packed S-RBC (1 ml) were suspended in 20 ml of barbiturate buffer adjusted to pH 10.0 with 1.3 ml of 1.4 N NaOH, and sensitized by adding 600 mg of KPG. The mixture was incubated at room temperature for 1 hr, and the sensitized cells were then washed three times in 0.01 M Tris buffer at pH 8.2, as described by Levine et al. (12).

Eagle's medium (2 ml) containing 0.7% of Noble agar, KPG-S-RBC (0.1 ml), and spleen cell suspension (0.2 ml) were mixed and poured into a standard petri dish. After incubation for 1 hr at 37 C, the plates were flooded with 1.5 ml of commercial (Baltimore Biological Laboratory) guinea pig complement (diluted 1 in 4) and reincubated for an additional 1 hr at 37 C. Hemolytic plaques were enumerated by use of a Quebec counter.

Chlorphenesin in concentrations greater than 0.1% (w/v) in physiological saline usually caused hemolysis of S-RBC and, for this reason, the effect of higher concentrations of the drug in the agar could not be studied by the LHG technique.

RESULTS

Chlorphenesin, at a final concentration of 0.1% (w/v), when added to the agar used to plate spleen cells from mice that were immunized with S-RBC, reduced the counts of hemolytic plaques by 50 to 70% (Table 1). Chlorphenesin reduced the plaque counts to a similar degree when added to the assay agar that contained spleen cells from mice immunized with the penicillin G-Keyhole limpet hemocyanin conjugate (BPO·KLH) (Table 2). Thus, it appeared that the action of chlorphenesin to reduce the number of plaques was equally effective in two different antigen-antibody systems.

The effect of chlorphenesin added to the spleen cell suspension prior to plating was investigated next. In these experiments, spleen cells from immunized animals were incubated with 0.1% chlorphenesin for 30 or 60 min at 37 C prior to plating. The results were compared with those obtained when chlorphenesin in the same concentration was added to the Jerne plates at the time of plating. The drug in concentration of 0.1% usually did not have detectable cytotoxic effects on the spleen cells, as judged by the appearance of the trypan blue-stained cells under the microscope.

The effect of adding 0.1% chlorphenesin to a spleen cell suspension prepared from spleens of mice immunized with S-RBC is presented in Table 3. The results indicated that the incubation of the spleen cell suspension with chlorphenesin for 1 hr at 37 C prior to plating reduced the plaque count very significantly, and was much more effective in this respect than was the addition of similar concentrations of chlorphenesin to the plates.

In these experiments, different concentrations of chlorphenesin were used, and the effect of incubating spleen cell suspensions from immunized animals for 1 hr in these solutions was studied. A dose-response relationship between the concentration of chlorphenesin and the number of plaques became obvious (Table 4).

TABLE 1. *Effect of chlorphenesin in agar on S-RBC antibody plaque formation*

Chlorphenesin (mg/ml)	Plaques per 10 ⁸ spleen cells ^a
0	8,197
1	1,767

^a Mean of duplicate plates made from pool of five spleen cell suspensions. Mice were immunized for 3 days prior to spleen removal.

TABLE 2. *Effect of chlorphenesin in agar on penicillin antibody plaque formation*

Treatment	Per cent chlorphenesin	Avg no. (±SE) of plaques per 10 ⁸ nucleated cells ^a
Control (untreated)	0	30 ± 8.5
	0.1	14 ± 3.0
BPO·KLH ^b	0	1,423 ± 273
	0.1	426 ± 116

^a Average of five spleens.

^b Penicillin G-Keyhole limpet hemocyanin conjugate.

TABLE 3. *Effect of incubation of spleen cell suspension with chlorphenesin before plating*

Chlorphenesin concentration (mg/ml)	Duration of incubation (min)	Avg no. of plaques per 10 ⁸ nucleated cells ^a
0	0	2,232
0	60	1,720
1	0	1,628
1	30	698
1	60	139

^a Average of five spleens.

The stronger the concentration of chlorphenesin in which the spleen cells were incubated prior to plating, the greater was the reduction of the number of plaques. These effects may not be due to cytotoxic action of chlorphenesin, because the number of viable nucleated spleen cells was not reduced by the drug.

Mode of action of chlorphenesin. There are several ways in which the addition of chlorphenesin to plates with spleen cells from immunized animals could reduce this complement-dependent antigen-antibody reaction. Chlorphenesin may coat the S-RBC, and thus prevent their reacting with the antibody (hemolysin). Chlorphenesin may exert its effects by destroying complement or by preventing the interaction of complement with the antigen-antibody complex. Finally, chlorphenesin may affect the antibody

TABLE 4. *Effect of chlorphenesin concentrations in spleen cell suspensions from mice immunized with S-RBC*

Chlorphenesin (mg/ml)	Plaques per spleen after incubation for 1 hr at 37 C ^a	Nucleated spleen cells ($\times 10^8$) per spleen after incubation
0	10,120	2.3
0.05	3,880	3.0
0.1	2,820	2.4
0.5	1,490	2.4
1.0	790	2.4

^a Mean of duplicate plates made from pool of five spleens.

or antibody release. Experiments were conducted to evaluate these possibilities.

In order to determine whether chlorphenesin blocked the reaction of S-RBC with hemolysin, S-RBC were incubated in 0.1% chlorphenesin for 1 hr at 37 C, and subsequently were washed three times in physiological saline. The use of these cells in Jerne plate assays did not cause any significant reduction in the number of hemolytic plaques (Table 5). Thus, it appeared that chlorphenesin either did not "bind" to the S-RBC or it did not block the interaction of hemolysin with the S-RBC.

The effect of chlorphenesin may be due to the inactivation of complement. To evaluate this possibility, chlorphenesin was added directly to the guinea pig sera that was used as the source of complement. The addition of 0.1% (w/v) or of 0.5% of chlorphenesin to guinea pig sera and incubation at 5 C or at 25 C for 1 hr did not diminish their complement activity (Table 6).

In another experiment to determine whether chlorphenesin affects the interaction of complement with the antigen-antibody complexes, spleen cells from mice immunized with S-RBC were plated with S-RBC and incubated for 1 hr at 37 C to permit release of hemolysin and interaction with the antigen. Thereafter, 2 ml of a 0.1% chlorphenesin solution were overlaid on the agar surface and incubated for 30 min at 37 C. The chlorphenesin solution was then poured off, and the agar surface was washed twice with physiological saline. The complement solution was then overlaid and plates were reincubated for 1 hr. The hemolytic plaque counts of the plates treated with chlorphenesin were similar to the counts of plates that were not treated with chlorphenesin (Table 7). These results indicate that chlorphenesin did not interfere with the interaction of complement with the antigen-antibody complex. An experiment similar to the one described above was also performed in which the plates were flooded with complement

TABLE 5. *Effect of chlorphenesin pretreatment of indicator sheep red blood cells prior to plating*

Chlorphenesin (mg/ml)	Plaques per 10^8 spleen cells ^a
0	6,107
1	5,820

^a Mean of duplicate plates made from pool of five spleen cell suspensions. Mice were immunized for 3 days prior to spleen removal.

TABLE 6. *Effect of chlorphenesin on complement*

Per cent chlorphenesin added to complement solution	Avg no. hemolytic plaques per 10^8 nucleated spleen cells ^b	
	Not incubated	Incubated 1 hr at 5 C
None ^a	4,790	5,580
0.10	5,115	6,045
0.50	3,255	4,650

^a Control.

^b Average of two plates from pools of five spleens.

TABLE 7. *Effect of chlorphenesin added to Jerne plates 1 hr after the S-RBC and hemolysin and prior to addition of complement*

Amount of chlorphenesin (mg/ml) added after antigen-antibody interaction	Plaques per 10^8 spleen cells ^a
0	7,873
1	7,398

^a Mean of duplicate plates made from pool of five spleen cell suspensions. Mice were immunized for three days.

without previous removal of chlorphenesin. The results obtained were similar, and indicated again that chlorphenesin did not destroy complement and that it did not block the interaction of the hemolysin and S-RBC complex with complement.

DISCUSSION

It has been noted previously that certain chemicals can affect the plaque formation in Jerne plates. Jerne and Nordin (9) reported that plaque formation did not occur in the presence of 0.01 M potassium cyanide. Berenbaum (2), studying the mechanism of this effect, concluded that agents usually regarded as metabolic inhibitors, such as cyanide or 2,4-dinitrophenol, may cause inhibition of plaque formation by their direct effect on antibody, perhaps by splitting disulfide bonds or by cleaving peptide bonds adjacent to

the cystine amino group. Fitch et al. (7), in collaboration with Berenbaum, found that potassium cyanide reduced plaque formation by acting directly on cells and not on antibody.

Chlorphenesin may also inhibit plaque formation by having a direct effect on the cell. This effect, however, would have to be of a different nature than that produced by substances such as cyanide. Chlorphenesin, in contrast to metabolic inhibitors, is a relatively nontoxic substance closely related to the widely used skeletal muscle relaxant, mephenesin (3). Like mephenesin, it produces in animals reversible paralysis of voluntary muscles by a depressant action on the interneurons of the spinal cord. The very large doses of the drug needed to produce these effects, which are of the order of 300 mg per kg intraperitoneally for mice, and their complete reversibility, would hardly qualify chlorphenesin to be described as a metabolic inhibitor.

Becker and Pisciotta (1) reported that spleen cells from rats immunized with S-RBC, when incubated with certain phenothiazines prior to plating, markedly increased the number of hemolytic plaques in Jerne plates. They interpreted this effect to be the result of increased cell membrane permeability produced by these compounds. Chlorpromazine and other phenothiazines have hemolytic properties in vitro which are similar to those of chlorphenesin. It is thus of great interest that the phenothiazines should affect the immune spleen cells in an opposite direction from chlorphenesin.

When suitable concentrations of chlorphenesin (0.1%) were added to the agar plates, a significant reduction of hemolytic plaques was noted not only with penicillin but also with the S-RBC system. Thus it appears that this effect of the drug is not specific for penicillin, and that other antigen-antibody systems such as the S-RBC-hemolysin system may also be affected.

It appears of significance that the addition of chlorphenesin to spleen cell suspensions from immunized animals resulted in a much greater reduction in the number of hemolytic plaques than did the addition of the substance to the Jerne plates. The preincubation of the spleen cells with chlorphenesin did not, as a rule, cause any detectable reduction in the number of viable, nucleated spleen cells. This effect suggested that chlorphenesin in some way affects the antibody itself or its ability to react with antigen (5). It is also possible that chlorphenesin may suppress

the antibody release from antibody-forming cells. These possibilities are now being investigated.

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