STUDIES ON DIRECT COOMBS REACTION BY CEFAZOLIN *IN VITRO*

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Cefazolin was tested *in vitro* for its reactivity in the direct COOMBS test as compared to other related antibiotics. Minimal concentrations of the antibiotics which were required to produce a direct COOMBS reaction were 2.5 mg/ml for cephalothin, 5 mg/ml for benzyl penicillin, 10 mg/ml for cephaloridine, and 40 mg/ml for cefazolin. Cefazolin even at this high concentration gave only a weak positive reaction and this depended upon the COOMBS serum employed. The intensity of the positive reaction was apparently related to that of the direct antibiotic-induced lytic action on the red blood cells used (cephalothin > benzyl penicillin > cephaloridine > cefazolin). These positive reactions are believed to arise from the binding of normal globulin, directly, or otherwise indirectly through antibiotics, to the surface of red blood cell walls which have been impaired by the antibiotics. It seems therefore that these phneomena *in vitro* are not immunological in nature.

The COOMBS test, also named the antiglobulin test, is widely used to detect blood-group antibodies absorbed on the surface of red blood cells. The occurrence of COOMBS-positive hemolytic anemia in patients receiving prolonged treatment with high dosages of penicillins by the intravenous route has been reported by several workers.[1,2,3,4,5,6,8] PERKINS[11], MOLTHAN[12], GRALNICK[13], YORK[14] and KAPLAN[15] have reported that not only penicillins but also cephalosporins (cephalothin or cephaloridine) caused positive COOMBS reactions when given intravenously in large dosages.

This paper reports some of the results of a study of the effect of cefazolin,[16,17,18,19] a newly developed cephalosporin antibiotic, on the direct COOMBS test *in vitro*. Cefazolin was tested *in vitro* for its reactivity in the direct COOMBS test as compared to other related antibiotics.

Materials and Methods

1. Antibiotic used

Cefazolin (CEZ, Fujisawa Research Laboratories), cephalothin (CET, Eli Lilly and Co.), cephaloridine (CER, Glaxo Laboratories) and benzyl penicillin (PC-G, Fujisawa Research Laboratories).

2. COOMBS sera used

Midori's anti-human globulin serum (Lot. 34), Ortho's anti-human globulin serum

(Lot. R-7454-4), Takeda's anti-human globulin serum (Lot. 36), and HYLAND's goat anti-rabbit γ-globulin serum (Lot. 8232H001A1) were each obtained from commercial sources.

3. Blood samples uesd

Normal human blood of types O and A, normal white rabbit blood.

4. Anticoagulants

EDTA and ALSEVERS solution were employed.

5. Performance of the direct COOMBS test *in vitro*

The blood to be tested (20 ml) was mixed with an equal volume of ALSEVERS solution, or alternately the blood after adding EDTA (20 mg) was diluted with an equal volume of saline solution (0.85 %).

One ml of the diluted blood was added to an equal volume of normal saline containing varying concentrations of the antibiotic to be tested. The reaction mixtures were incubated at 37°C for 3 hours with gentle shaking. The red blood cells (RBC's) were collected by centrifugation and washed with saline (10 times) by alternate cycles of suspension and centrifugation to remove excess antibiotic and non-adsorbed serum proteins. The washed cells were diluted to a 2 per cent suspension (volume per volume) with normal saline solution and this preparation is termed the "antibiotic-coated RBC suspension". To each drop of one of these "antibiotic-coated RBC suspension" there was added one drop of Midori's anti-human globulin serum, 2 drops of Ortho's anti-human globulin serum or one drop of Takeda's anti-human globulin serum. Immediately afterwards, the suspensions were centrifuged at 1,000 r.p.m. ($80 \times g$) for one minute. The resultant packed cells were gently agitated and then arbitrarily scored as +++, ++, +, ± and −, depending on the degree of agglutination observed. The direct COOMBS test was conducted similarly using the rabbit blood and goat anti-rabbit-γ-globulin serum (HYLAND).

6. Direct lytic action of antibiotics on RBCs

Heparinized red cells, obtained from normal human blood type O and washed 3 times with sufficient volumes of saline to remove the plasma proteins, were made up to a 20 % suspension in saline. To each 2 ml of this suspension was added an equal volume of varying concentrations of the antibiotic solution. The mixtures were incubated at 37°C for 3 hours with gentle shaking, and centrifuged at 1,500 r. p. m. for 10 minutes. The absorbance of the supernatant was measured at 541 mμ to determine the hemolytic value. The same procedure was followed with rabbit RBCs.

Results

1. Effect of antibiotics on direct COOMBS reaction of normal RBCs *in vitro*

(1) Human whole blood

The results are summarized in Table 1. Two anticoagulants, ALSEVERS solution and EDTA, and commercially available anti-human globulin serum from different suppliers were used.

The concentrations of cephalothin required for producing a positive *in vitro* test were 2.5 mg/ml or more. The concentrations of other antibiotics required to produce a positive test under the same conditions were 5~10 mg/ml of benzyl penicillin and 10~20 mg/ml of cephaloridine, respectively. However, cefazolin required concentrations higher than those found for any other agent; negative reactions were obtained at concentrations as high as 40 mg/ml using Midori's serum, the test was weakly positive at 20 mg/ml using Ortho's serum (EDTA), and also weakly positive at 40 mg/ml using Takeda's serum. The kinds of anticoagulants and COOMBS sera used affected to an extent the concentration required for positive results.

Table 1. Direct Coombs test of antibiotic-coated human whole blood cells

Antibiotic	Final conc. (mg/ml)	Coombs' reagents					
		Midori		Ortho		Takeda	
		Alsevers	EDTA	Alsevers	EDTA	Alsevers	EDTA
Cephalothin (CET)	40	++	++	+	+++	+++	+++
	20	++	++	+	++	++	++
	10	++	++	+	+	+	++
	5	+	+	±	+	±	+
	2.5	−	−	±	±	−	±
	1.25	−	−	−	−	−	−
Benzyl penicillin (PC-G)	40	+++	++	+	++	+	+
	20	++	++	+	+	±	+
	10	+	++	±	+	±	±
	5	−	−	−	±	±	±
	2.5	−	−	−	−	−	−
	1.25	−	−	−	−	−	−
Cephaloridine (CER)	40	++	++	+	++	+	±
	20	+	+	±	±	±	±
	10	±	−	±	±	±	±
	5	−	−	−	−	−	−
	2.5	−	−	−	−	−	−
	1.25	−	−	−	−	−	−
Cefazolin (CEZ)	40	−	−	−	±	±	±
	20	−	−	−	±	−	−
	10	−	−	−	−	−	−
	5	−	−	−	−	−	−
	2.5	−	−	−	−	−	−
	1.25	−	−	−	−	−	−

(2) Rabbit whole blood

The results are presented in Table 2. Goat anti-rabbit γ-globulin serum free from rabbit blood cell antibodies was used as the Coombs serum. The rabbit whole blood gave negative results in the direct Coombs test *in vitro*, although the human whole blood caused a positive reaction as mentioned before.

(3) Washed human RBCs

Human RBCs of type A were washed with saline to remove all traces of plasma protein and were finally suspended in saline at their original concentration. After adding varying concentrations of an antibiotic solution, the suspension was incubated at 37°C for 3 hours. The resultant antibiotic-coated RBCs were washed to remove any unbound antibiotic. The direct Coombs test was performed on this washed antibiotic-coated RBCs suspension, to which the original serum was also added in order to examine its effect on the reaction. The results are shown in Tables 3, 4 and 5.

Table 2. Direct Coombs test of antibiotic-coated rabbit whole blood cells

Antibiotic	Concentration of antibiotic (mg/ml)						
	20	10	5	2.5	1.25	0.63	0.31
CET	−	−	−	−	−	−	−
PC-G	−	−	−	−	−	−	−
CER	−	−	−	−	−	−	−
CEZ	−	−	−	−	−	−	−

Table 3. Direct Coombs test of antibiotic-coated human whole blood cells

Antibiotic	Concentration of antibiotic (mg/ml)						
	40	20	10	5	2.5	1.25	0.63
CET	+++	+++	++	+	+	±	−
PC-G	++	+	+	±	−	−	−
CER	+	±	±	−	−	−	−
CEZ	±	−	−	−	−	−	−

Table 4. Direct Coombs test of prewashed antibiotic-coated human red blood cells

Antibiotic	Concentration of antibiotic (mg/ml)						
	40	20	10	5	2.5	1.25	0.63
CET	−	−	−	−	−	−	−
PC-G	−	−	−	−	−	−	−
CER	−	−	−	−	−	−	−
CEZ	−	−	−	−	−	−	−

Table 5. Direct Coombs test of prewashed antibiotic-coated human red blood cells which exposed to original serum

Antibiotic	Concentration of antibiotic (mg/ml)						
	40	20	10	5	2.5	1.25	0.63
CET	+++	+++	+++	++	±	−	−
PC-G	+++	++	+	±	−	−	−
CER	++	+	−	−	−	−	−
CEZ	±	−	−	−	−	−	−

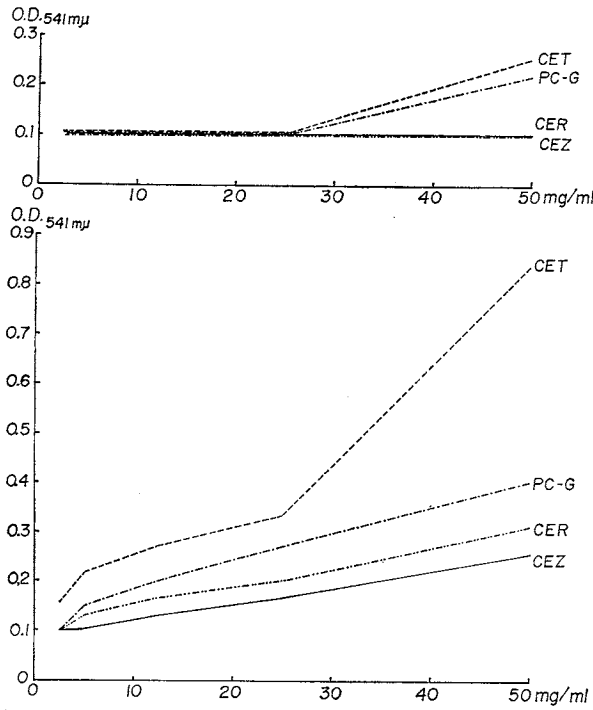
In the case of normal human whole blood (type A), the direct Coombs test became positive at concentrations above 1.25 mg/ml for cephalothin, 5 mg/ml for benzyl penicillin, 10 mg/ml for cephaloridine and 40 mg/ml for cefazolin.

However, with serum-free washed antibiotic-coated RBCs, none of the antibiotics caused a positive Coombs reaction. The addition of human serum to the serum-free washed antibiotic-coated RBCs caused positive reactions at the above-mentioned concentrations of antibiotics.

2. Direct lytic action of antibiotics on RBCs

As shown in Fig. 1, cephalothin and benzyl penicillin caused direct lysis of human RBCs at a concentration of 50 mg/ml. On the other hand, cefazolin and cephaloridine did not produce lysis at this concentration. Hemolysis of rabbit RBCs by the direct lytic action of antibiotics was more pronounced than that of human RBCs (Fig. 1). The strength of the lytic action of the antibiotics used was: cephalothin>benzyl penicillin>cephaloridine>cefazolin.

Fig. 1. Direct lytic action of antibiotics on human RBCs (upper figure) and direct lytic action of antibiotics on rabbit RBCs (lower figure)



Discussion

It has been known that in patients receiving large intravenous doses benzyl penicillin can cause acute immunohemolytic anemia with a positive direct Coombs reaction. This untoward phenomenon is considered to be due to a circulating penicillin antibody (IgG).[3,5,6,8] However, the immunologic mechanism of the final RBC destruction is still obscure. In fuadine-induced anemia, the patient's serum agglutinates normal RBCs in the presence of the drug. Shulman, using the equilibrium dialysis technique, found that the antibody alone has little affinity for RBCs. Accordingly, Shulman and others have pro-

posed that the antibodies combine specifically with the drug to form a stable complex, which is further absorbed by the surfaces of RBCs and induces the characteristic drug induced reaction.[7,9,10] Recently, GRALNICK *et al.*[13] and MOLTHAN *et al.*[12] reported that the direct COOMBS test became positive when cephalosporins were added to normal human blood *in vitro*. Our present experiment was performed according to the method of GRALNICK *et al.* and MOLTHAN *et al.*, and we are able to confirm their results with respect to cephalothin and cephaloridine. However, higher concentrations of these antibiotics were required than in these earlier experiments. In contrast to our results, MOLTHAN *et al.* did not obtain positive COOMBS reaction with benzyl penicillin.

The following findings were of particular interest in our experiments: (1) The strength of the positive direct COOMBS reaction was related to that of the direct antibiotic-induced lytic action on the RBCs of rabbit or man. (2) Exposure of the washed RBCs to antibiotics did not cause a positive direct COOMBS reaction. The antibiotic-coated RBCs obtained were washed to remove residual antibiotics, and then exposed to human serum. A positive direct COOMBS reaction occurred therefrom. This finding is apparently inconsistent with the hypothesis of MOLTHAN and others, which states that the positive direct COOMBS reaction is caused by the combination of antibiotic-binding plasma protein pre-existing in the blood with the surface of the RBCs. Our studies indicate that the positive direct COOMBS reaction is due to the binding of normal globulin, directly, or otherwise indirectly, through antibiotics, to the RBCs surfaces that have been impaired by the antibiotics. Rabbit RBCs, unlike the human RBCs, produced no positive direct COOMBS reaction. Whether this is due to the repulsive property of the impaired RBCs surfaces to the globulin, or to the preferred binding of globulins other than γ-globulin, remains obscure, because of the fact that the antiglobulin serum used represents an antibody against γ-globulin. The significance of this reaction as a cause of hemolytic anemia is not clear at this time.

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