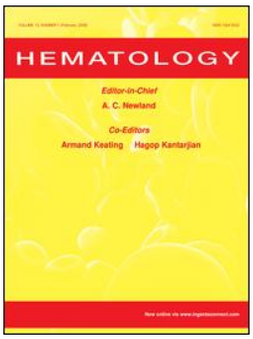

Hematology

ISSN: (Print) 1607-8454 (Online) Journal homepage: https://www.tandfonline.com/loi/yhem20

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To cite this article: G. Malaponte, C. Arcidiacono, C. Mazzarino, S. Pelligra, G. Li Volti, V. Bevelacqua & S. Li Volti (2000) Cephalosporin-induced Hemolytic Anemia in a Sicilian Child, Hematology, 5:4, 327-334, DOI: 10.1080/10245332.2000.11746527

To link to this article: https://doi.org/10.1080/10245332.2000.11746527

Hematology, 2000, Vol. 5, pp. 327–334
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Erythropoiesis

Cephalosporin-induced Hemolytic Anemia in a Sicilian Child*

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(Received 8 March 2000; In final form 22 May 2000)

A 27-month-old child developed acute hemolysis on two occasions after the administration of cephalosporin. On the first occasion, hemolysis was intravascular and was due to the formation of complexes between antibodies and the drug, which bound to red blood cells and caused severe hemolysis. On the second occasion, hemolysis was extravascular and was probably due to antibody-dependent cell-mediated cytotoxicity. Marked increases in levels of CD^+_{19} and CD^+_{57} CD^+_8 cells were detected among the subpopulations of the patient's lymphocytes but only in the level of CD^+_{19} cells from the patient's father, after incubation of a sample of whole blood with a solution of cephalosporins. These results might explain the differences between the immune response of the patient and those of other members of his family and of an unrelated control.

Keywords: Hemolytic anemia, drug-induced hemolysis, cephalosporins, antibody-dependent cell-mediated cytotoxicity, direct Coombs test, indirect Coombs test

INTRODUCTION

Several antibiotics, including penicillin, tetracycline, cephalosporins, rifampin and sulfonamides, can induce the hemolysis of red blood cells (RBCs) [1] by any or all of the following mechanisms: (i) the antibiotic can adsorb to membranes of RBCs forming a new antigen that reacts with a specific IgG, with resultant extravascular hemolysis [2,3]; (ii) the antibiotic can induce the formation of antibodies and then complexes of these antibodies with the antibiotic can bind to RBCs, with resultant intravascular haemolysis as a consequence of the activation of complement [2,4]; (iii) the antibiotic can adsorb proteins non-immunologically from the plasma and then antibiotic-coated RBCs, upon contact with normal plasma can adsorb albumin,

*Part of this paper was published in the European Journal of Haematology, as a Letter to the Editor (Vol. 62, 362–363, 1999).

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immunoglobulins and complement [5], with resultant extravascular hemolysis; and (iv) formation of auto-antibodies and acute hemolysis results from attack by the immune system [6–8]. Nonetheless, clinically recognisable hemolysis occurs very rarely [9], in spite of the fact that highly variable percentages of individuals (3–80%) have been reported to yield positive results in a direct Coombs' test (DCT) [10] after the administration of cephalosporin.

We report again the case of a child who, after the administration of cephalosporin, experienced two episodes of acute hemolysis [11]. The first episode was characterized by intravascular hemolysis that was due to the activation of complement secondary to the formation of immunocomplexes. In the second episode, the hemolysis was probably due to the adsorption of the drug to RBC membranes. In order to strengthen this last hypothesis, we assayed the antibody-dependent cell-mediated cytotoxicity (ADCC) using autologous components (RBCs, effector cells and serum) of the patient, his parents, his brother and of an unrelated control.

CASE REPORT

Hemolysis occurred in a young boy of 27 months of age, who had suffered from recurrent infections of the upper respiratory tract. These infections had usually been treated with ampicillin but, when he was 18-month-old, he was treated just once with cefaclor. When he was 21 months old, he was admitted to a pediatric hospital because of sudden pallor and jaundice observed three days after the administration of cefixime (10 mg/kg/day for three days; 350 mg in total) and niflumic acid (570 mg/day for two days; 1,140 mg in total). Apart from pallor (Hb, 9.5 g/dl; RBCs, $3.5 \times 10^6/mm^3$; Ht, 28.4%)and jaundice (direct bilirubin, 0.7 mg/dl; total bilirubin, 5.5 mg/dl; haptoglobin, 30 mg/dl) at admission, his physical examination was unremarkable. Hemoglobinuria was present but erythrocyte glucose-6-phosphate dehydrogenase (G6PhD) activity during the course of the hemolytic crisis was normal. The anemia worsened rapidly and four days after the admission the hematological data were as follows: Hb, 5.3 g/dl; RBCs, $2.6 \times 10^6/mm^3$ and Ht, 16.1%, and the reticulocyte count was 20%. No blood transfusion was given since he quickly improved. Thus, the patient was discharged with a diagnosis of drug-induced hemolytic anemia. Three months later, because of fever and dysphagia, the child was inadvertently given intramuscular cefotaxime (50 mg/kg/day; 750 mg in total) by the family doctor but for just one day since his parents noticed pallor and mild jaundice. There was no hemoglobinuria by the test strips and the patient made a rapid recovery. Recently, the patient was admitted to the Pediatric Department for additional investigations. At admission, the physical examination was unremarkable and results of routine laboratory tests (RBC, WBC, Hb, Ht, reticulocyte count, erythrocyte G6PhD activity, bilirubin and urinalysis) were normal. Results of a DCT and indirect Coombs' test (ICT) were negative. High levels of circulating immunocomplexes were found.

METHODS

Serological Evaluation

Examinations *in vitro* of erythrocytes and serum were performed according to the methods described by the American Association of Blood Banks [2,12] not only from the patient and a unrelated control but also from his parents and his brother in the attempt to assess a possible inheritance. DCT and ICT were performed with polyvalent rabbit antisera against human IgG, IgA, IgM, C_3 and C_4. For ICT, we used cephalosporin-coated or uncoated 0DCcee group RBCs. The eluate, obtained as described by Rubin [13], and the corresponding serum were diluted 1:20 with phosphate-buffered saline (PBS) and

incubated with compatible and previously coated or uncoated RBCs [14] that were allowed to react with the serum or the eluate for 30 min at 37 °C. After three washes with PBS, polyvalent antibodies against human globulins were added. DAT was performed with specific antisera. The degree of agglutination was scored from 0 to 4 +.

Antibody-Dependent Cell-Mediated Cytotoxicity

Assays of ADCC *in vitro* were performed using autologous components (RBCs, effector cells and serum).

Preparation of Effector Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood by centrifugation on gradient of a Ficoll-Hypaque (Pharmacia, Cologno Monzese, Italy) as described by Boyum [15]. Monocytes were purified from PBMCs by centrifugation on an isosmotic solution of 46% Percoll (Pharmacia), as described by Mantovani *et al.* [16]. The monocytes were collected from the interface between gradient and blood, washed three times with PBS and resuspended in modified RPMI 1640 (Gibco BRL Life Technologies, S. Giuliano Milanese, Italy) that contained 10% fetal calf serum (Hy Clone, Europe Ltd, Erembodegem-Aalst, Belgium), 2mM L-glutamine (Gibco BRL Life Technologies) and 50 μg/ml Gentamycin (Hy Clone, Europe Ltd.). The purity of monocytes was examined on a FACScanTM flow cytometer (Becton-Dickinson, Mountain View, CA, USA) with monoclonal antibodies specific for CD14 and CD11c, (Becton-Dickinson). Cell viability was determined by the trypan blue exclusion test and revealed that 95% of cells were viable.

Preparation of Target Cells

Four hundred mg of each cephalosporin (cefaclor, cefixime and cefotaxime) were dissolved in 10 ml of barbital buffer (pH 9.6). One ml of a suspension of 10 × washed RBCs was incubated for 1 h at 37 °C with the cephalosporins (40 μg/ml). The RBCs were then washed four times with PBS and an aliquot of RBCs was labelled with $Na_2{}^{51}CrO_4$ (NEN Life Science Products, Cinisello Balsamo, Italy). After labelling, target cells were washed four times, resuspended in complete medium (RPMI 1640), monitored for μ-radioactivity and incubated for 30 min at room temperature with autologous serum or eluate. An aliquot was then incubated with Ig-specific antiserum (Caltag Laboratories, Burlingame CA, USA), treated as above described and used immediately for the cytotoxicity test.

Cytotoxicity Test

Cytotoxicity was measured by a modified version of the standard 4-hour ^{51}Cr-release [14,17]. ^{51}Cr-labelled RBCs, coated or uncoated with cephalosporins, were added to each well of V-bottomed 96-well microtiter plates (NUNC Life Technologies, Milano, Italy) together with a sample of the serum, eluate or PBS. Effector cells were then added in triplicate to yield various ratios of effector to target (E:T) cells, which ranged from 20:1 to 1:1. The plates were incubated for 4 h at 37 °C in a humified atmosphere of 5% CO_2. Then the cell suspension was centrifuged at 400 × g for 4 min and 100 μl of the supernatant was removed and radioactivity was determined as an index of the amount of isotope release. The percentage of specific lysis for each target was calculated as follows:

$$\frac{\text{experimental release} - \text{spontaneous release} \times 100}{\text{maximum release} - \text{spontaneous release}}$$

where the maximum release was that from target cells incubated with 1% sodium dodecyl sulfate (SDS) and spontaneous release was that from target cells incubated in medium alone.

Subsets of Lymphocytes

We determined the relative sizes of subpopulations of lymphocytes before and after incubation with cephalosporin for the patient, his parents, his brother and a healthy control. Venous blood from each individual was collected in EDTA and analyzed immediately according to the guidelines provided by GIC [18].

RESULTS

Table I shows the results of the ICT of serum from the patient, his parents, his brother and an unrelated control, after incubation for 1h at 37 °C with compatible and previously cephalosporin-coated RBCs. The ICT gave a moderately positive result with the patient's sample and a weakly positive result with that of his father.

Table II shows the results of the ICT for the patient, his parents, his brother and the control after incubation for 1 h at 37 °C when serum, cephalosporin and complement had been incubated with compatible and uncoated RBCs. The ICT gave a strongly positive result with the patient's seurm, and a weakly positive result with that of the father.

Table III shows the percentages of hemolysis observed when PBMCs and purified monocytes were allowed to react with RBCs from each subject. Moderate hemolysis (13.8%) was observed when PBMCs that had been incubated with the patient's serum were allowed to react with cephalosporin-coated RBCs. Mild hemolysis (3.2%) was observed when we used the patient's RBC eluate. Greater hemolysis was observed when we used purified monocytes as effectors cells and cephalosporin-coated RBCs, incubated with serum (36.6%) and with the eluate (17.7%) from the patient. Limited hemolysis was observed when the father's PBMCs (4.1%) and purified monocytes (6.3%) were allowed to react with autologous serum and cephalosporin-coated RBCs. The incubation of the father's target cells with the eluate and autologous effector cells did not induce hemolysis, and no hemolysis occurred when the father's eluate was allowed to react with autologous RBCs from the control. No hemolysis was observed with RBCs, serum or eluate from the mother and the brother of the patient.

TABLE I Results of the indirect Coombs' tests performed with serum incubated with compatible and antibiotic-coated RBCs

	Source of serum				
Antibiotic	Patient	Father	Mother	Brother	Control
Ampicillin	—	—	—	—	—
Cefaclor	++−	±−	—	—	—
Cefixime	++−	±−	—	—	—
Cefotaxime	++−	±−	—	—	—

++− moderately strong reaction; ±−, weak reaction; −, no reaction.

TABLE II Results of the indirect Coombs' tests performed with serum, antibiotics, complement and PBS incubated with compatible RBCs

	Source of RBCs				
Antibiotic	Patient	Father	Mother	Brother	Control
Ampicillin	—	—	—	—	—
Cefaclor	+++	+−	—	—	—
Cefixime	+++	+−	—	—	—
Cefotaxime	+++	+−	—	—	—

+++, strong reaction; +−, moderate reaction; −, no reaction.

Figure 1 shows trends in the patient's ADCC test and their dependence on the E:T ratio. When results of the purified monocytes were incubated with autologous serum and cephalosporin-coated RBCs the greatest hemolysis was observed at the highest value of E:T (20:1), and when the E:T ratio was reduced there was an exponential decrease in the percentage of hemolysed cells. Similar trends, although with lower percentages of hemolysed cells were obtained when ADCC test was performed with PBMCs as effector cells. By contrast, when we used uncoated RBCs as target cells no hemolysis was observed at any E:T ratio tested.

Figure 2 shows the relative sizes of populations CD_{19}^{+} and $CD_{57}^{\pm}$ CD_{8}^{+} cells (all other

TABLE III Antibody-dependent cell-mediated cytotoxicity (ADCC) reactions against autologous RBCs

Target RBCs*	Source of antibodies*	Effector cells*	% immune lysis (child)	% immune lysis (father)
Coated	—	PBMCs	0.4	−1.9
Coated	—	purified monocytes	0.7	−1.1
Coated	Patient's serum	PBMCs	13.8	4.1
Coated	Patient's serum	purified monocytes	36.6	6.3
Coated	RBC eluate	PBMCs	3.2	−2.4
Coated	RBC eluate	purified monocytes	17.7	−1.5
Coated	Normal serum	PBMCs	−2.7	−3
Coated	Normal serum	purified monocytes	−1.4	−1.8
Uncoated	—	PBMCs	−3.4	−3.6
Uncoated	—	purified monocytes	−2.3	−3
Uncoated	Patient's serum	PBMCs	−2.6	−2.8
Uncoated	Patient's serum	purified monocytes	−1.8	−2.3
Uncoated	RBC eluate	PBMCs	−2.1	−2.4
Uncoated	RBC eluate	purified monocytes	−0.8	−1.6
Uncoated	Normal serum	PBMCs	−2.4	−3.2
Uncoated	Normal serum	purified monocytes	−1.1	−2.1

*RBCs, antibody and effector cells were obtained from the blood of the patient; the serum and the RBC eluate were diluted 20:1; normal serum was diuted 1:1.

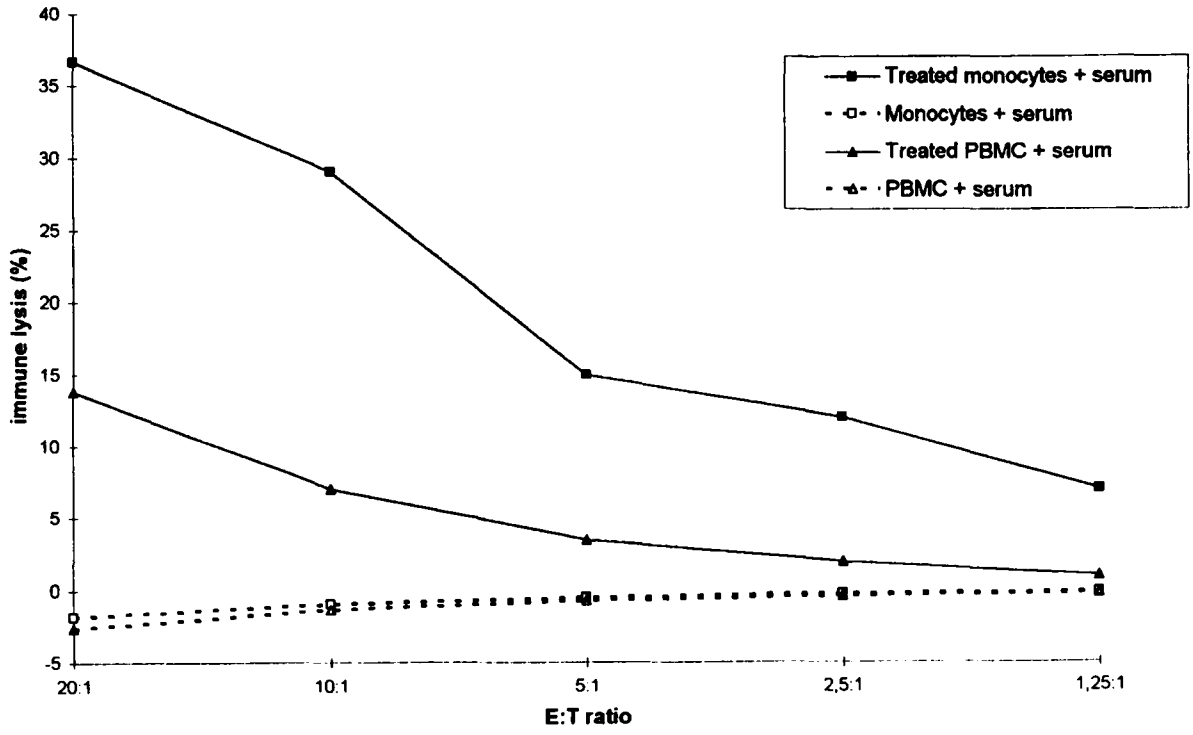

FIGURE 1 Lysis of cells in the antibody-dependent cell-mediated cytotoxicity (ADCC) test and its dependance on the E:T ratio.

subsets of lymphocytes were normal) before and after incubation of whole blood at 37 °C for 1 h with cephalosporins, for the patient and his father. Marked increases in levels of CD_{19}^{+} cells were observed in the case of both the father and the patient after the incubation. In addition, a significant increase in the level of $CD_{57}^{+}CD_{8}^{+}$ cells was observed only in the case of the patient.

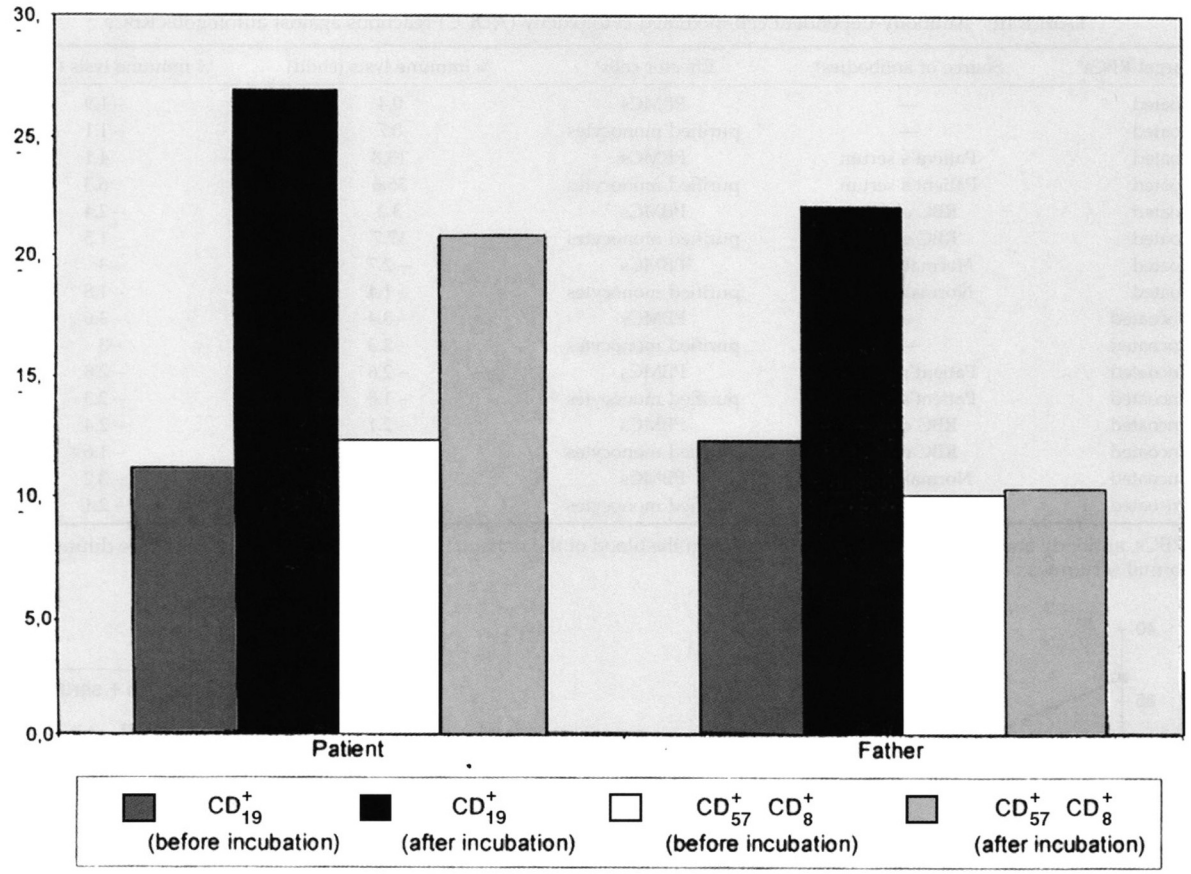

FIGURE 2 Percentages of CD_{19}^{+} and CD_{57}^{+}/CD_{8}^{+} cells obtained before and after incubation with a solution of cefixime.

The relative sizes of the subpopulations of lymphocytes in the patient's mother, in his brother and in the unrelated control before and after incubation of whole blood at 37 °C for 1 h with cephalosporins were unchanged.

DISCUSSION

Clinical and laboratory data demonstrated that our patient had experienced two episodes of acute hemolysis, which were due to different pathogenetic mechanisms, after the administration of cephalosporin. In the first episode, hemolysis was intravascular (as indicated by the presence of hemoglobinuria) and was induced by the formation of immune complexes, as demonstrated by the fact that the patient's serum with cephalosporin strongly reacted with compatible RBCs when complement was added (Table II). The ICT gave a strongly positive result in the case of patient and a weaker positive result in the case of his father. By contrast, the second episode of hemolysis was extravascular and hemoglobinuria was absent. Extravascular hemolysis can be due to an ADCC reaction after the production of specific antibodies against cephalosporins that were adsorbed to the surface of RBCs. Hemolysis of autologous and cephalosporin-coated RBCs occurred *in vitro* when the patient's monocytes were allowed to react with autologous serum or eluate, demon-

strating that an ADCC-related mechanism was involved when extravascular hemolysis occurred [17]. This reaction was highly specific since a negative result was obtained when we used uncoated RBCs from the patient. Moreover, when we used purified monocytes as effector cells, the cytotoxicity increased markedly. In fact, purified monocytes were specific effectors of the ADCC reaction.

To our knowledge, this is the first report of a case in which two distinct mechanisms precipitated two separate episodes of cephalosporin-induced hemolysis in a single patient. Chambers *et al* [19], Shulman *et al* [20] and Maraspin *et al* [21] reported patients in whom cephalosporin-induced hemolysis was due to the actions of two distinct pathogenetic mechanisms that operated simultaneously. We cannot exclude the possibility that the first episode of hemolysis might also have been due to two distinct pathogenetic mechanisms that were operating simultaneously. It remains to be determined why in the second episode there was no hemoglobinuria. A shorter exposure to the drug probably triggered hemolysis only at the extravascular level.

Another question arises from the results of our present analysis. The ICT, as well as the testes of ADCC of PBMCs and purified monocytes, gave a weakly positive result for the patient's father. However, unlike his son, he had never developed acute hemolysis after the administration of cephalosporin. Whatever the pathogenetic mechanisms in our patient, he alone, among all members of his family, was able to couple cephalosporins to RBCs and to mount an exaggerated immune response to such drugs. In our analysis, we demonstrated a marked increase in the relative level of CD_{19}^{+} cells both in our patient and in his father and an increase in the relative level of CD_{57}^{+} CD_{8}^{+} cells in the patient. The latter subset of lymphocytes is known to have cytolytic activity and, thus, it might have played a role in the pathogenetic mechanism of acute hemolysis. The more dramatic phenotypic expression in our patient than in his father might have been due to interference and effects, in the patient, of modifier genes [22] inherited from his mother.

In conclusion, care must be taken in the use of antibiotics to avoid drug-induced anemia. In fact, cross-reactivity of common epitopes with additional types of antibiotic might increase the incidence of severe acute hemolytic anemia.

Acknowledgments

We are greatly indebted to Prof. Rino Giustolisi for his precious suggestions.

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