CHAPTER I

INTRODUCTION

The administration of drugs may lead to the development of a wide variety of hematologic abnormalities. Some of these abnormalities involve red blood cells and may be caused by an immunological mechanisms (1). Patients often present with positive direct antiglobulin (Coombs) test, antibody in their sera and may be associated with a hemolytic anemia.

The first drug that was proven to cause a positive direct antiglobulin test and immune hemolytic anemia was Fuadin (Stibophen). In 1954 (2), and 1956 (3), Harris was the first who gave a complete report of an example of the first mechanism of acquried hemolytic anemia secondary to drug administration. He described a patient, who when treated for schistosomiasis with Fuadin, developed acute intravascular hemolysis. The patient's serum was shown to contain a factor which strongly agglutinated his own or normal red blood cells. These reactions were active only when Fuadin was present in the reacting system.

Since then many other drugs which can cause a positive direct antiglobulin test and immnue hemolytic anemia have been described. Many of these drugs were reported from single patient i.e., quinine, chlorpromazine, and dipyrone (Table 1). Penicillin and alpha methyldopa were by far the most coomon drugs to cause immune hemolytic anemia.

Table 1 Drugs that have been reported to cause a positive direct antiglobulin test and hemolytic anemia.

Drug	Year First described	No. of Patients reported	References
Fuadin (Stibophen)	1954	3	2, 3, 31
Quinidine	1956	2	32, 39
P-amino salicylic acid (PAS)	1956	4	34, 43
Quinine	1958	1	33
Phenacetin	1958	3	33, 34
Penicillin	1959	18	7-11, 24-26
Insecticides (Chlorinated hydrocarbons)	1959	1	45
Isonicotinic acid hydrazide (Isoniazid)	1960	1	46
Chlorpromazine	1961	1	40
Dipyrone	1966	1	41
Alpha-methyldopa (Aldomet)	1966	144	55-62
L-phenylalanine mustard (Melphelan)	1967	1	47
Cephalothin (Keflin)	1967	3	28, 29
Mefenamic acid (Ponstel)	1968	3	48, 49
Carbromal (Carbrital)	1970	3	30
Sulfonylurea derivatives	1970	4	50, 51
Insulin	1970	1	52
L-dopa	1971	2	68, 69
Rifampicin	1972	1	53
Tetracycline	1974	1	54

The pathogenesis of red blood cells sensitization by drug-related antibody with or without fixation of complement is variable and there is a relationship between the responsible drug, the mechanism of red cell sensitization, clinical manifestation and laboratory methods of diagnosis (4). There seems to be at least four mechanisms by which the drugs produce hemolytic anemia with a positive direct antiglobulin test. The first mechanism is typified by penicillin; the second, by stibophen; the third, by alpha-methyldopa; and the fourth, by cephalothin.

Mechanism of the positive direct antiglobulin test and possible hemolytic anemia due to drugs

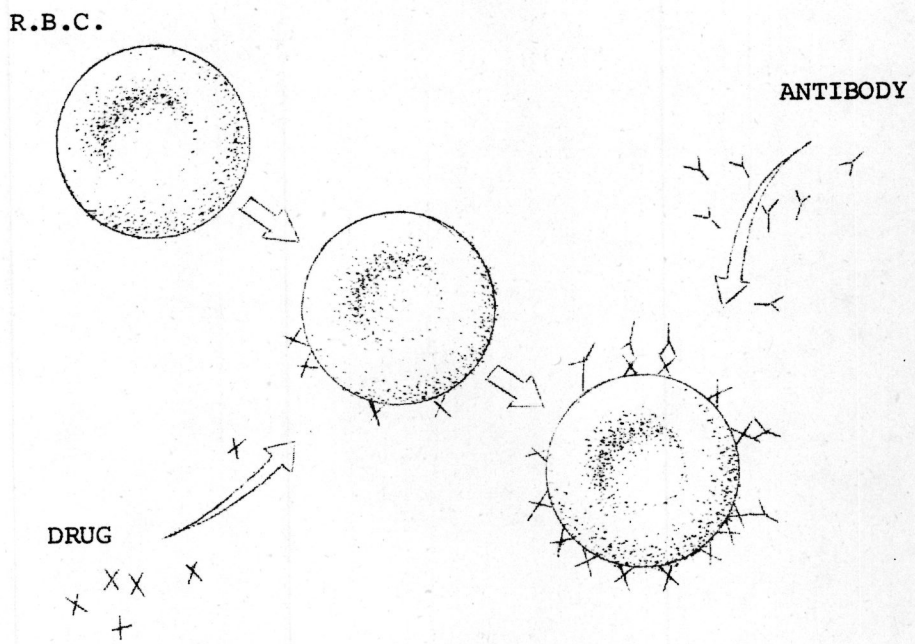
1. Penicillin type, drug adsorbed onto red blood cells.

Circulating antipenicillin antibody (CAPA) was first reported by Ley et al in 1958 (5). During routine blood bank testing, a serum of a proselective transfusion recipient was found to agglutinated the red blood cells of 25 group O members of an antibody idendification panel. This red blood cells panel had been stored in a preservative solution containing penicillin. The same red blood cells panel, when not exposed to penicillin were not agglutinated by this serum. Since the original described of antipenicillin antibody by Ley et al the administration of penicillin to patient with CAPA to cause the positive direct antiglobulin and possibly hemolytic anemia had been described (6-12)

The mechanism of the positive direct antiglobulin test and hemolytic anemia in patients receiving massive doses of intravenous penicillin seems clear (9,11). The drug is adsorbed to the red blood

cells and the anti-penicillin antibody present in the patient's plasma will react with the penicillin on the red blood cells. The end product is a red blood cell senisitzed with IgG (Figure 1).

Figure 1 Mechanism of development of positive direct antiglobulin test caused by penicillin



Penicillin in one of the few drugs that binds firmly to protein. Penicillin will combine with the proteins on normal red blood cell membranes both *in vivo* and *in vitro*. The drug cannot be removed even by multiple washes in saline. There is no fall in hemagglutination titer eventhough red blood cells sensitized with penicillin *in vitro* are washed twenty to twenty-five times with buffered saline solution one week period; studies with tritium labeled penicillin also indicated that penicillin derivative is "firmly bound" to the red blood cells (13).

This means that one can sensitized red blood cell with penicillin or preparing penicillin-treated red blood cell with penicillin and then use these treated red blood cells for the detection of anti-penicillin antibody

Specificity for the anti-penicillin antibody had been evaluated by hemagglutination inhibition using benzylpenicillin (Penicillin G) and a wide variety of penicillin derivatives. B enzylpenicillin itself appears not to be responsible for in vivo antigenic stimulation (13,14) or in vitro red blood cell sensitization. Evidence exist that the immunogenicity of penicillin is due to its ability to react chemically with tissue proteins to form several different haptenic groups (13-17). The major haptenic determinants is the benzylpenicilloyl (BPO) group.

Levine and Redmond (11) suggested from their data that BPO specific Coombs positivity results from the coupling of BPO groups of red blood cell surfaces by chemical reaction with penicillin in vivo as a primary event, followed by specific binding of BPO antibody from the plasma. In these situations, the quantity of BPO specific antibody coating the red blood cell would be limited by the number of BPO haptenic groups on red blood cell, the plasma concentration of BPO specific antibodies, and the avidity of binding of antibodies to BPO coupling red blood cell. However, the antibody specificity found on testing by hemagglutination inhibition reactions indicates multiple antigenic determinants in different patients (13-17).

Levine, Fellner and Levytska (16) subsequently showed that over 90% of patients selected at random had BPO specific antibodies. These antibodies in most patients belong to the immunoglobulin M (IgM), are

inhibited by BPO hapten (15-16). On the other hand, the antibodies found in patients with penicillin induced hemolytic anemia are immunoglobulin G (IgG) (9-15). In 1966, Petz and Fudenberg (9) defined the causative role of penicillin induced Coombs positive hemolytic anemia. They described the unusual characteristics of the anti-penicillin antibody in their patient who developed hemolytic anemia as compared with previously characterized antibodies in patients without hemolytic anemia by others and are summerized in Table 2. The antibody was atypical in that it was almost entirely IgG, and was difficult to inhibit *in vitro* with benzylpenicillin and penicillin derivatives.

Table 2 Characteristics of Circulating Anti-penicillin Antibody

Characteristic No.	Description
1	May act *in vitro* by direct agglutination or indirect Coombs test using penicillin-sensitized red blood cells (5, 19, 20).
2	May be eluted from antibody coated red blood cells (5, 21).
3	May be responsible for positive direct Coombs test during penicillin administration (6, 7, 9).
4	Is active over wide range of temperature (4°-37°C) (21).
5	Can be absorbed from serum by penicillin sensitized red blood cell (21, 22).

Characteristic No.	Description
6	Is stable at 56°C for up to 6 hrs.
7	Antibody activity by indirect Coombs test resides in gamma G globulin; hemagglutinin usually gamma M globulin (13, 14, 15, 19).
8	Antibody usually easily inhibited by benzylpenicillin and a variety of penicillin derivatives (8, 15, 20)

There is no direct correlation between the presence of IgG and IgM penicillin hemagglutinating antibodies and allergic reactions (18,19, 20). Most workers have found no correlation at all, but a few have found that high titer IgG antibodies occur more often in the allergic group (20). Perhaps a better correlation will be found with IgE penicillin antibodies.

Characteristics of penicillin induced hemolytic anemia are as follows :'

1.1 The administration of a very large doses of penicillin (at least 10 million units per day for a week or more).

1.2 A high titer IgG penicillin antibody is present in the serum

1.3 The direct antiglobulin test is strongly positive due to sensitization with IgG. Rarely, complement components are detected as well, and complement activation may contribute to the immune hemolysis (12, 24)

1.4 Antibody eluted from the patient's red blood cells will ract only against penicillin-treated red blood cells.

1.5 Hemolytic anemia is usually less acute in onset than that

caused by drugs of group 2 but may be life threatening if the etiology is unrecognized and penicillin administration is continued.

1.6 Cessation of penicillin therapy is followed by complete recovery, but hemolysis of decreasing severity may persists for several weeks.

1.7 Other manifestations of penicillin allergy are not necessarily present.

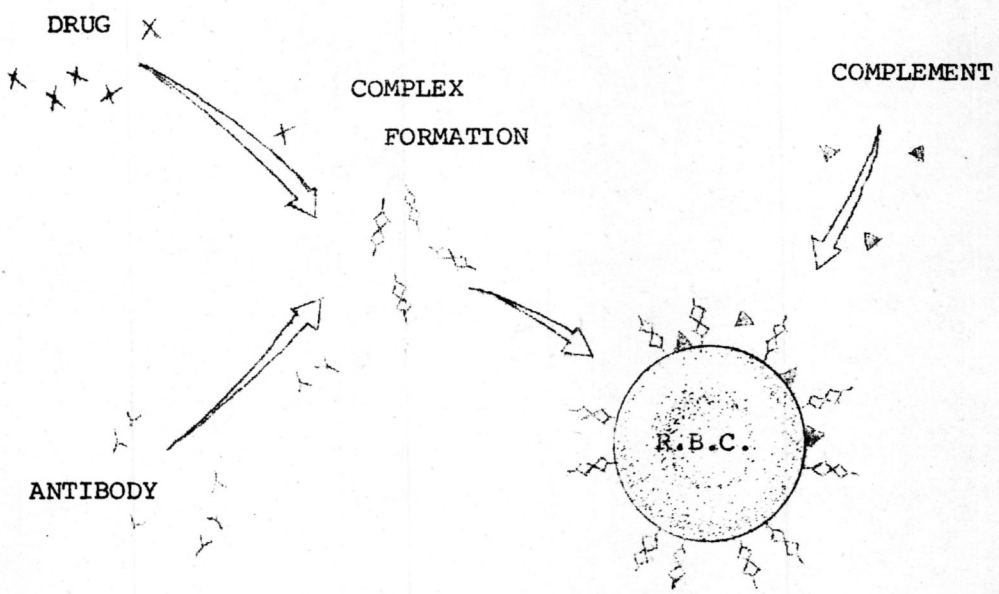
Other drugs known to bind firmly to red cells and cause positive direct antiglobulin test by the same mechanism as penicillin are cephalothin (27-29) and possibly carbromal (30). Cephalothin (28) has been described as a rare cause of immune hemolytic anemia but carbromal so far has not.

2. Stibophen type, immune complex adsorption to red blood cells.

Several drugs though chemically unrelated can bring about injury of red blood cells, platelets or leukocytes by a similar immunologic mechanism. Among these drugs are stibophen (Fuadin) 2, 3, - , 31), quinidine (32), quinine, phenacetine (33, 34) and Sedormid (35). In his earlier work on Sedormid purpura, Ackroyd (35), proposed that drug may act as a hapten, combining loosely with the cell membrane and stimulating antibody production to the combined antigen. This concept would not differ greatly from the mechanism just discussed above for penicillin induced immune hemolysis. Shulman (36) proposed a different mechanism which is accepted by most investigators as the most probable explanation for the reactions seen with all the drugs mentioned in Table 1 except penicillin, cephalosporin, alpha-methyldopa, L-dopa and possibly carbromal. Shulman extended the findings of Miescher and

co-workers (37) by showing that drugs such as quinine, quinidine and stibophen have a far stronger affinity for its antibodies than the affinity of the drugs for the cell membrane. From this and other evidence, Shulman proposed that when drug, antibody and red blood cell were present together, as they would be in circulation of the patient, the first reaction was the formation of antidrug antibody complex. This immune complex will be adsorbed to the red blood cell membrane, often activating complement in the process (Figure 2). This resulted either in red blood cell lysis or in an irreversible coating of the red blood cell with complement proteins. The antidrug antibody complex then appears to dissociate spontaneously from the damaged red blood cell and

Figure 2 Mechanism of development of positive direct antiglobulin test caused by drugs such as phenacetin.



to be free to react with other red blood cells. Thus, a relatively small number of complexes would be capable of injuring a very large number of blood cells in vivo. The dissociation of the antidrug antibody complex from the red blood cell explains failure to detect any immunoglobulin on the red blood cell surface.

From the observation of many investigators, it is of interest that one patient developing antibodies to a given drug, for example, quinidine, may develop thrombocytopenia (38). Another patient with quinidine sensitivity may have hemolytic anemia (39). A rare patient (1), may show both platelet and red blood cell involvement. Shulman (36) found that the antibody determining red blood cell injury in such cases is regularly a 19S globulin and that the antibody determining platelet injury is characteristically a 7S globulin but he was not further described. So it is unknown why this immune complex once formed sometimes cause red blood cell destruction only and other times platelet destruction oly.

The most common characteristics of this group of drugs in causing immune abnormalities are as follows:

2.1 The patient needs to take only a small quantity of the drug.

2.2 "In vitro" reactions are only observed when patient's serum, drug and red blood cells are all incubated to gether.

2.3 The serum antidrug antibody is often IgM and capable of activating complement.

2.4 Renal failure is frequent.

2.5 The direct antiglobulin test in positive, often due to

presence of complement components on the red blood cell surface, usually without detectable immunoglobulins. This may explain that the immune complex does not bind firmly to the red blood cells and may dissociate from the red blood cells and be free to react with other cells. Furthermore, red cells sensitization by IgM antibodies is not readily detectable by the antiglobulin test (42).

2.6 Acute intravascular hemolysis with hemoglobulinemia and hemoglobulinuria is the usual clinical presentation (16 from 19 reported cases). (81).

3. The alpha-methyldopa (Aldomet) type, red blood cell autoantibodies induced by drugs (unknown mechanism).

In 1966, Carstairs, Worlledge and co-workers (56) reported the occurrence of the positive direct antiglobulin test and autoimmune hemolytic anemia in three hypertensive patients, all of whom had been taking alpha-methyl-3, 4-dihydroxy-L-phenylalanine (methyldopa, Aldomet) for over one year. These findings led them to survey 104 patients being treated for hypertension. Fifty-seven of 104 were taking methyldopa, and five were found to have a positive direct antiblogulin test with anti-IgG serum. None of these patients with a positive direct antiglobulin test was anemic, and all had been taking methyldopa for over one year. In comparison, none of the 47 remaining patients taking other anti-hypertensive medications had a positive direct antiglobulin test. Subsequent to this report, letters were published (57-60) regarding a hemolytic anemia occurring in six additional patients who were taking methyldopa. In all the reported cases, the direct anti-

globulin test was positive, but only with anti-IgG serum. Hayes (59) could find no antibodies in her patient's serum using saline, albumin and indirect antiglobulin test. An attempt to demonstrate antibody by elution was unsuccessful. Hamilton et al (58) detected free antibody in the sera of two patients receiving methyldopa. Free antibody in one of their patients, serum reacted with all red blood cells except those of rare Rh type, D-, and in another patient the antibody reacted more strongly with adult red blood cells than with fetal red blood cells.

Carstairs and co-workers (61) reported a larger survey of 202 consecutive hypertensive patients taking methyldopa, 41 of whom had a positive direct antiglobulin test. None of 41 patients demonstrated frank hemolytic anemia, although six had mildly elevated reticulocyte counts and one of four had a shortened red blood cells life span. The incidence of positive direct antiglobulin test was higher in patients receiving larger doses of the drug, and in most instances the test become positive between six and twelve months after starting the treatment with methyldopa.

At the same time, Worlledge, Carstairs and Dacie (62) reported on 25 of 30 patients who developed a hemolytic anemia while receiving methyldopa. Their serologic findings were identical to those found in idiopathic autoimmune hemolytic anemia. The direct antiglobulin test was positive, and in most cases the abnormal autoantibodies present had clear Rh-specificity. The antibodies seemed not to be directed against the alpha methyldopa or its derivatives.

The immunologic mechanism in this form of hemolytic anemia are distinct from those of penicillin induced hemolytic anemia and from the "innocent bystander" mechanism seen with quinidine or stibophen. This is suggested by the occurrence of antibodies reacting not with alpha-methyldopa but with the normal red blood cells. The antibodies reacted similarly to those seen in the idiopathic autoimmune hemolytic anemia, in that they often showed some Rh blood group specificity (62).

The precise role of alpha-methyldopa in the pathogenesis of red blood cell autosensitization is at present unclear althouth two theories have been suggested :

A. Adsorption of drug to red blood cells : Wurzel and Silverman (64) showed that normal washed red blood cells and red blood cells in plasma would adsorb alpha-methyldopa and that if normal whole blood was incubated in a high concentration (e.g., 0.5 mg/ml) of drug for several days. In vitro, then a positive antiglobulin test was obtained. Gottlieb and Wurzel (65) recently demonstrated that alpha-methyldopa or one of its metabolites could be covalently bound to gamma globulin when drug and plasma were incubated together for 2 days or more. If red blood cells were then added, a positive antiglobulin test occurred. No Rh specificity was noted. Other workers (62, 66) have not been able to demonstrate a positive antiglobulin test following incubation of red blood cells in alpha-methyldopa or its derivatives. Furthermore, Lo Buglio and Jandl (66) demonstrated that alpha-methyldopa had very little affinity for plasma proteins or red blood cell membranes, although it should be noted that the incubation period employed was shorter than

used by Gottlieb and Wurzel (65).

B. Altered autoantigens : Worlledge et al (62, 63) have postulated that the drug or one of its metabolites may combine with the red blood cell membrane or enter the red blood cell and in some way alter the Rh antigens so that the normal immune system no longer recognizes them as "self". The incorporation may occur at the normoblast or reticulocyte stage, thus explaining the delay in developing of a positive direct antiglobulin test.

Two other drugs have recently been described as acting in a similar fashion to alpha-methyldopa. One is a closely related drug L-dopa, which was described as causing positive direct antiglobulin test in about 6 to 9% of the patients receiving the drug (68). Recently, this drug has been reported as a cause of hemolytic anemia (69)

The other drug, mefenamic acid, is unrelated to methyldopa and has been described as causing positive direct antiglobulin test and autoimmune hemolytic anemia in two separate reports of five patients (48, 49).

The clinical and laboratory characteristics of alpha-methyldopa induced abnormalities are as follows :

3.1 Indistinguishable in laboratory from "idiopathic" warm type autoimmune hemolytic anemia.

3.2 Positive direct antiglobulin test found in 15% of patients receiving methyldopa (report vary from 10% to 36%). Incidence seems to vary between racial groups, being highest in Cancasian, lower in Chinese and almost absent in Blacks (75).

3.3 Positive direct antiglobulin test due to sensitization with IgG, and all patients with hemolytic anemia have a very strongly positive direct antiglobulin test.

3.4 Direct antiglobulin test usually becomes positive after three to six months of treatment.

3.5 The development of the positive antiglobulin test appears to be dose-dependent. About three times as many patients (36%) giving positive tests when taking more than 2 g of the drug daily compared with 11% of patients taking less than 1 g daily, 19% became positive on 1 to 2 g daily.

3.6 Reports vary from 0 to 5% of patients receiving methyldopa having evidence of hemolytic anemia. The cumulative evidence is 0.8%

3.7 The positive direct antiglobulin reaction gradually becomes negative once methyldopa is stopped. This may take from one month to two years. Fortunately, the clinical and hematological values improve very rapidly.

4. Cephalothin type, modification of red blood cell membrane by drug allowing nonimmunological absorption of protein.

The development of positive direct antiglobulin test, serum antibodies and possibly hemolytic anemia during cephalothin administration have been reported by several investigators (70-73).

Three mechanisms for the positive direct antiglobulin test due to cephalothin have been proposed :

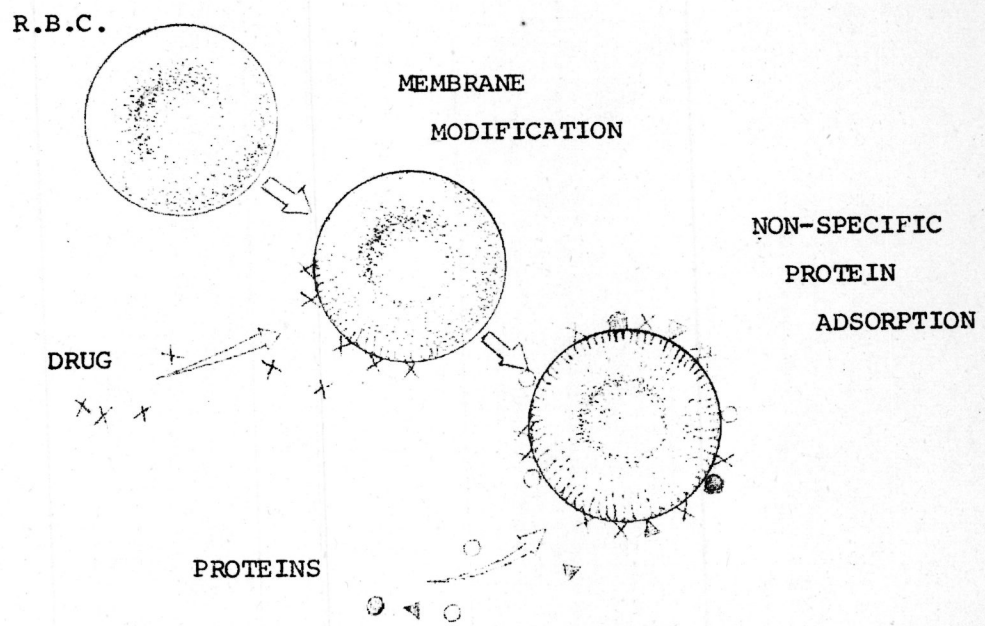
4.1 The red blood cell membrane may be modified by the drug so that the red blood cell now takes up proteins nonimmunologically (28, 29,

70).(Figure 3). Cephalothin sensitized red blood cells incubated in normal plasma in vitro become coated with IgG and other serum proteins.

4.2 The drug may combine with the red blood cell membrane, as does penicillin, and being so closely related chemically to penicillin, cross-reacting penicillin antibodies will now combine with the drug (26, 27, 70-73).

4.3 The drug may combine with the red blood cell membrane and a specific anticephalothin antibodies in the serum, which may react with drug on the red blood cell membrane in the same fashion to penicillin (28, 70).

Figure 3 One of the mechanisms of development of a positive direct antiglobulin test caused by cephalothin.



Cephalothin, like penicillin, will bind firmly to red blood cells. These red blood cells can be washed many times *in vitro* without the drug being removed. Cephalothin-treated red blood cells can be used in a similar fashion to penicillin-treated red blood cells in the detection of cephalothin or penicillin cross-reacting antibodies (71).

Spath *et al* (71) were able to confirm and extend the finding of Gralnick (27), Nesmith (26), Abraham and co-workers (78) in showing that penicillin antibodies may cross react with cephalothin-treated red blood cells; thus high titer penicillin antibodies may react with cephalothin-treated red blood cell both *in vivo* and *in vitro*. In addition, specific anticephalothin antibodies exist, and these can sometimes be shown to be present in eluates from red blood cells demonstrating a positive direct antiglobulin test due to cephalothin (28, 70).

Immune hemolytic anemia following cephalothin administration is rare. Gralnick *et al* (28) reported two patients who developed Coombs' positive hemolytic anemia while receiving cephalothin sodium therapy, a specific anticephalothin antibody was demonstrated, and the antibody directed against cephalothin appeared to be IgG. They also noted that the clinical setting of hemolytic anemia associated with cephalothin seems to differ from that of penicillin-induced hemolytic anemia. With the penicillin, the hemolysis usually associated with the administration of large doses of penicillin and hemolysis is not apparent within the first week of therapy. In contrast, their patients received relatively normal doses of cephalothin sodium (2 and 6 g), and hemolysis was noted within one week after cephalothin therapy had been initiated.

Cephalothin and penicillin-induced hemolytic anemia appear to run similar clinical courses, which hemolysis persisting for weeks and months after the drug has been discontinued.

Other drug closely related to cephalothin such as cephaloridine (72), and cefazolin (73) have also described as a cause of positive direct antiglobulin tests.

The purpose of this study is to determine the effect of antidrug antibody to red blood cells of patients receiving penicillin and alpha-methyldopa. These groups of patients were selected for this study because both of drugs are by far the most common drugs to cause positive direct antiglobulin test and hemolytic anemia. In this study the incidence of positive direct antiglobulin test, the characteristics and titers of antipenicillin antibodies were determined, with an attempt to define the hematologic, chemical and immunohematologic correlations of the positive direct antiglobulin test.