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AUTOIMMUNITY—EXPERIMENTAL AND CLINICAL ASPECTS

PART II

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CONTENTS: PARTS II

SECTION V. PANEL DISCUSSION

Hemolytic Anemia. *By* J. V. DACIE 415

Autoimmune Hemolytic Disease: Observations of Serological Reactions and Disease Activity. *By* R. S. EVANS, MARGARET BINGHAM AND ELIZABETH TURNER 422

Clinical Correlations of the Direct Antiglobulin Reaction. *By* SCOTT N. SWISHER, NORMA TRABOLD, JOHN P. LEDDY AND JOHN VAUGHAN 441

The Structure and Specificity of Erythrocyte Autoantibodies. *By* BERNARD PIROFSKY 448

The Specificity of the Antibody in Paroxysmal Cold Hemoglobinuria (P.C.H.). *By* PHILIP LEVINE, M. J. CELANO AND F. FALKOWSKI 456

Acquired Hemolytic Anemia. *By* ERNEST WITEBSKY 462

Some Comments on Autoantibody Induced Red Cell Destruction. *By* J. J. VAN LOGHEM 465

Experimental Production in Man of Autoantibodies with Rh Specificity. *By* JAMES F. MOHN, REGINALD M. LAMBERT, HERBERT S. BOWMAN AND F. WELLS BRASON 477

Current Problems of Chronic Cold Hemagglutinin Disease. *By* H. SCHUBOTHE .. 484

Reaction of I131 Trace Labeled Cold Agglutinin. *By* MORTEN HARBOE 491

Similarities between Known Antiplatelet Antibodies and the Factor Responsible for Thrombocytopenia in Idiopathic Purpura. Physiologic, Serologic and Isotopic Studies. *By* N. RAPHAEL SHULMAN, VICTOR J. MARDER AND ROY S. WEINRACH 499

Acute "ITP" in Isoimmunized Dogs. *By* MARIO BALDINI 543

SECTION VI

Autoantileukocyte Ribosomal Fraction in Leukoneutropenias. *By* J. DAUSSET .. 550

Cytotoxicity of Pathologic Human Sera for Lymphocytes and Neutrophils. *By* ROY L. WALFORD, ROBERT GALLAGHER AND PAT TAYLOR 563

Antigenicity and Autoantigenicity of Collagen. *By* C. STEFFEN 570

Thyroiditis. *By* WILLIAM H. BEIERWALTES 586

Autoimmunity in Pernicious Anemia and Thyroiditis: A Family Study. *By* D. DONIACH, I. M. ROITT AND K. B. TAYLOR 605

Cytotoxicity of Human Thyroid Autoantibodies. *By* JOSEPH H. KITE, JR., NOEL R. ROSE, KYOICHI KANO AND ERNEST WITEBSKY 626

Autoimmunity in Pernicious Anemia and Atrophic Gastritis. *By* I. M. ROITT, D. DONIACH AND C. SHAPLAND 644

The Clinical and Pathological Significance of Gastric Parietal Cell Antibody. *By* W. J. IRVINE, S. H. DAVIES, S. TEITELBAUM, I. W. DELAMORE AND A. WYNN WILLIAMS 657

Autoimmunity in Iron Deficiency Anemia. *By* J. H. DAGG, A. GOLDBERG, J. R. ANDERSON, J. S. BECK AND K. G. GRAY 692

SECTION VII

Autospermagglutinis: A Cause of Infertility in Men. *By* PHILIP RÜMKE 696

Autospermagglutinins: Immunofluorescent Studies. *By* T. E. W. FELTKAMP, KATINKA KRUYFF, NITA C. J. J. LADIGES AND PHILIP RÜMKE 702

Serologic Studies of Myasthenia Gravis. *By* ERNST H. BEUTNER, ERNEST WITEBSKY AND AIDA Y. DJANIAN 709

Studies in Myasthenia Gravis: Immunofluorescent Tagging of Muscle Striation with Antibody from Serums of 256 Myasthenic Patients. *By* KERMIT E. OSSERMAN AND LEE BURNETT WEINER 730

Immunological Concomitants of Myasthenia Gravis. *By* ARTHUR J. L. STRAUSS, HUGO W. R. VAN DER GELD, PIERSON G. KEMP, JR., E. DUBOIS EXUM AND HOWARD C. GOODMAN 744

Autoimmune Hepatitis. *By* IAN R. MACKAY, SARA WEIDEN AND JANET HASKER 767

Immune Processes in the Pathogenesis of Liver Disease. *By* HANS POPPER, FIORENZO PARONETTO AND FENTON SCHAFFNER 781

Partial Purification and Characterization of an Extractible Nuclear Antigen which Reacts with SLE Sera. *By* HALSTEAD R. HOLMAN 800

Cytoplasmic Antibodies in Patients with Systemic Lupus Erythematosus. *By* G. WIEDERMANN AND P. A. MIESCHER 807

Studies on Antinuclear Antibodies. *By* MAXIME SELIGMANN, ARLETTE CANNAT and MONIQUE HAMARD 816

The Nature of Antinuclear Immunoglobulins. *By* E. J. HOLBOROW AND G. D. JOHNSON 833

The Transfusion of Active LE Plasma into Nonlupus Recipients, with a Note on the LE-Like Cell. *By* ALBERTO M. MARMONT 838

SECTION VIII

The Frequent Occurrence of Hypergammaglobulinemia and Multiple Tissue Antibodies in Sjogren's Syndrome. *By* JOSEPH J. BUNIM 852

Observations on Antigamma Globulin Factors in Human Sera and Antibodies against Autologous Gamma Globulins Produced in Rabbits. *By* RALPH C. WILLIAMS AND HENRY G. KUNKEL 860

Some Aspects of the Relations between Rheumatoid Arthritis, Antigamma-Globulin Factors and the Polymorphism of Human Gamma Globulin. *By* R. GRUBB, G. KRONVALL AND L. MÅRTENSSON 865

The Heterogeneity of Rheumatoid Factors and the Genetic Control of Polypeptide Chains of γ-Globulins. *By* E. C. FRANKLIN AND H. H. FUDENBERG 873

Inhibition of Complement Fixation by Rheumatoid Sera. *By* RALPH HEIMER AND FRANCOISE LEVIN 879

Rheumatoid Factors and the Etiology of Rheumatoid Arthritis. *By* H. H. FUDENBERG AND E. C. FRANKLIN 884

Antinuclear Factors in Rheumatoid Arthritis. *By* E. V. BARNETT, J. P. LEDDY, J. J. CONDEMI AND J. H. VAUGHAN 896

Autoantibodies to Heart and Rheumatic Fever: The Induction of Autoimmunity to Heart by Streptococcal Antigen Cross-Reactive with Heart. *By* MELVIN H. KAPLAN 904

Discussion 916

Closing Remarks. *By* WILLIAM DAMESHEK AND ERNEST WITEBSKY 977

Section V. Panel Discussion

HEMOLYTIC ANEMIA

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The autoimmune hemolytic anemias easily hold their place amongst the increasing number of autoimmune disorders by virtue of their complexity and the many problems that still await solution. Historically, consideration of the role that antibodies might play in relation to anemia in experimental animals and man dates back to the early years of this century. But it was the introduction of the antiglobulin test in 1945 by Coombs, Mourant and Race that more than anything else was the starting-off point for the work which is to be discussed. In this introductory paper it is impossible for me to do more than to mention some general points and problems.

First, we cannot fail to be struck by the variability of the clinical syndrome of autoimmune hemolytic anemia. On the one hand, there are cases of so-called "idiopathic" origin: on the other, there are the cases associated in one way or another with an underlying disease. Autoimmune hemolytic anemia may, for example, be an important manifestation of the complex disorder systemic lupus erythematosus, occasionally it may be the dominant feature; it may develop during a patient's convalescence from virus pneumonia or at some stage during the evolution of chronic lymphocytic leukemia or reticulosarcoma, or it may be a presenting sign of an ovarian dermoid or teratoma. Then there is paroxysmal cold hemoglobinuria, which may, too, be apparently "idiopathic" in origin, secondary to syphilis or rarely may follow a virus infection. These so-called secondary (Watson, 1939) or symptomatic (Singer & Dameshek, 1941) cases represent a fascinating and important clinical problem. However, the exact relationship between the underlying diseases and the complicating hemolytic anemias is in most cases far from clear. It is unlikely to be the same in each type of case, but I have no doubt in my mind that these remarkable associations do provide important clues for the general understanding of autoimmune disease if we were but able to interpret them.

In my experience the majority of cases of autoimmune hemolytic anemias have not, however, developed on the basis of a clearly recognizable underlying disease and out of a total of 175 cases studied at the Postgraduate Medical School in London over a 14-year period, 115 or 71 per cent had to be classified as the "idiopathic" type.

Now, all patients who develop autoimmune hemolytic anemia, whether of the "idiopathic" or secondary type, form abnormal globulins which

are adsorbed to their red cells and/or circulate in their serum. This is the basis for the use of the prefix "autoimmune." The globulins vary greatly in their serological reactions from patient to patient, and a number of types of rather characteristic reaction patterns can be demonstrated in the laboratory.

There has been a good deal of discussion in the past as to whether the globulins should be looked upon as "true" antibodies and, if antibodies, whether they should be looked upon as autoantibodies. Now, however, their antibody nature is, I think, generally accepted, with the proviso that it is admitted that the coating of a red cell with protein giving rise to a positive antiglobulin reaction does not necessarily mean that the reacting substance is an antibody. It may, for instance, be complement adsorbed as a result of antibody activity (Dacie, Crookston & Christenson, 1957), or even, in theory at least, other nonantibody globulin adsorbed to cells which have sustained damage of one type or another. In relation to the non-γ-globulin antiglobulin reaction, I should add that recent work has identified at least two antibodies in the antiglobulin serum which react with components of complement. Thus, Harboe and his coworkers (1963) have identified anti-β_{1C} reacting with the C'3a antigen and anti-β_{1E} reacting with C'4.

The main points which identify coating globulins as antibodies include their transferability to normal red cells, the occasional presence in serum of similar globulins which can be adsorbed to normal cells and in many instances, as I shall refer to later, their ability to react with specifically identifiable red-cell antigens.

The antibodies may be characterized as "warm" or "cold," according to how temperature affects their activity, incomplete or complete, hemolytic and so on. They can also be classified according to molecular size, that is to say, they can be demonstrated to be 7S or 18-19S in type. Some types of antibody, such as incomplete warm antibodies, are relatively common; others such as complete (agglutinating) warm antibodies or hemolytic warm antibodies are rare. The common incomplete warm type of antibody seems always to be 7S (Fudenberg & Kunkel, 1957), and the common agglutinating but potentially hemolytic cold antibody is 18-19S (Gordon, 1953; Christenson *et al.*, 1957). The remarkable Donath-Landsteiner hemolytic cold antibody of paroxysmal cold hemoglobinuria is, on the other hand, 7S in type (Hinz, 1963).

The subtle differences which can be demonstrated *in vitro* in the serological behavior of different patients' antibodies are well recognized and are no doubt but a reflection of the uniqueness of the individual. One aspect of this patient-to-patient variability can be illustrated by reference to the different specificities of the antibodies, a problem about which a great deal of interest has been taken in recent years. In our own experience in about one-third of the patients forming warm antibodies it is pos-

sible to demonstrate a clear specificity for one or more Rh antigens, and Weiner and Vos (1963) have recently shown that if the apparent "non-specific" samples are tested against red cells showing various Rh gene deletion patterns, the majority of the patients' sera or eluted antibodies can be shown either not to react or to react weakly to various degrees with the deleted cells, suggesting that they, too, have a Rh specificity. It will be recalled that Wiener, Gordon and Gallop (1953) suggested that the autoantibodies in acquired hemolytic anemia might be directed against a hypothetical nucleus of the Rh-Hr substance.

The nature or specificity of the remaining antibodies which do not have any affinities with Rh antibodies is as yet unknown, I believe. Most do not appear to have any demonstrable specificity, and although it would clearly be rash to think that specificity will never be demonstrated, the concept of an antibody reacting indifferently with all human cells does not seem to me unreasonable.

When we come to the cold antibodies, then again we find that the reactions of most conform to a definite specificity pattern. The Ii specificity of Wiener and coworkers (1956) represents a peculiar blood-group system. The vast majority of adult individuals have I+ strongly reacting cells, and the great majority of high titer cold agglutinins are anti-I, in the sense that they react with normal adult cells strongly and with cord-blood cells or with the blood of the very rare adult i cells weakly. This is the typical reaction with the serum of patients suffering from the "idiopathic" type of the cold-hemagglutinin syndrome. But this is not true of many of the cold antibodies formed in association with lymphosarcoma and reticulosarcoma. Here the anti-I specificity seems often not to hold and two patients have been described who formed an antibody of antithetical specificity, anti-i (Marsh & Jenkins, 1960).

A recent further important discovery in the specificity field is that of Levine, Celano and Falkowski (1963). These authors showed that the Donath-Landsteiner antibody of paroxysmal cold hemoglobinuria has an unusual and unexpected specificity. This is against the Tj^a antigen. Thus the rare Tj^a-negative or *pp* cells are not hemolysed by this remarkable antibody. We ourselves have confirmed that is so in cases both of syphilitic and nonsyphilitic origin. Finally, I should like to mention antibodies which appear only to be very weakly adsorbed by normal cells but which are strongly adsorbed by, and powerfully agglutinate or hemolyse, enzyme-treated cells. Such antibodies are not uncommon, but their specificity if any is unknown and their significance is uncertain. Antibody components such as these seem to be of little importance from the point of view of clinical hemolysis. They may be present in high titers, yet the patients have a normal blood picture.

The reason for the peculiar specificity, directed so often against Rh antigens in the warm-antibody cases, is obscure, but the problem is an

intriguing one and the preference for Rh specificity must have some significance. Why for instance are autoantibodies not developed against anti-A or anti-B? Possibly these antigens are too powerful and widely distributed and immunological tolerance against them is thus very firmly based. Why, too, anti-Tj^a specificity in the case of Donath-Landsteiner antibody of paroxysmal cold hemoglobinuria and anti-I in the cold-hemagglutinin disease? Both these antibodies cross-react with animal red cells, and these antigens, or at least cross-reacting antigens, are thus widely distributed. The cold-agglutinin response in postvirus pneumonia hemolytic anemia has all the characteristics of a sharp and short-lived immune response and the same is true of the Donath-Landsteiner antibody response which rarely follows other virus infections. Low-titer cold autoagglutinins exist commonly as a natural, "normal," low-thermal amplitude autoantibody. The rise following virus pneumonia may thus be due to violent stimulation by cross-reacting antigens of the cellular clone producing an apparently normal antibody. Whether the Donath-Landsteiner antibody likewise normally exists in serum in very small amounts is not known.

More important perhaps than the niceties of *in vitro* serological reactions is the problem as to why and how the antibodies damage the red cells. First, it has to be admitted that they may apparently not always do so significantly, and all of us have probably met with apparently otherwise hematologically normal subjects, showing no signs of increased hemolysis, in whom the direct antiglobulin test is clearly positive. In some of these cases at least the antibody has been shown, I think, to be a 7S γ-globulin, capable of being eluted and transferred from the bearers' red cells to those of another normal subject, and reacting apparently in exactly the same way as does an incomplete antibody coating the red cells of a patient suffering from active hemolysis. Other types of antibody may be found occasionally in similar cases: a recent patient studied in London was found to have formed a complement-fixing antibody. Her red cells reacted strongly in the antiglobulin test with anticomplement sera and her serum strongly hemolysed enzyme-treated cells at 37°C., and very weakly, too, normal cells when the serum was suitably acidified. A ^{51}Cr red-cell survival study showed, however, no evidence of hemolysis.

These occurrences are puzzling, but it is certainly possible that they depend upon qualitative and quantitative differences in antibody activity, the state of equilibrium between antibody adsorbed to red cells and antibody free in plasma, and the effect that *p*H may have on this, etc. This is a broad field for speculation, observation and experiment.

I shall not discuss in any detail the way that hemolysis is brought about in cases where antibody formation is associated with clear evidence of hemolysis. The important researches of Jandl and his colleagues (Jandl, Richardson Jones & Castle, 1957; Jandl & Kaplan, 1960) and of Mollison (Cutbush & Mollison, 1958) have indicated the relative role of the spleen and

other reticuloendothelial organs in destroying red cells coated with incomplete, agglutinating or complement-fixing antibodies, respectively, and the role of autoagglutination and filter-pore size. Destruction is least rapid with incomplete antibodies, and unless very heavy amounts of antibody are being formed hemolysis occurs predominantly in the spleen. Patients forming incomplete antibody often in fact benefit from splenectomy, at least for a time. Agglutinating and complement-fixing antibodies lead to rapid red cell destruction widely in the reticuloendothelial organs of the body, particularly in the liver, and also to some extent, depending on the degree of sensitization, in the bloodstream.

I shall finish with a few remarks on etiology. Personally, I feel that several mechanisms are likely to be involved in the development of autoimmune hemolytic anemia in man. Somatic mutation leading to the development of forbidden clones of antibody-forming cells which are not susceptible to and thus escape from the normal homeostatic mechanisms which prevent autoantibody formation may be the explanation in many cases. The association of hemolysis with systemic lupus erythematosus (Pisciotta *et al.*, 1951) and ulcerative colitis (Fong, Fudenberg & Perlmann, 1963) suggests a predisposition on the part of some patients to develop autoimmune disease of more than one tissue—these patients may be looked upon perhaps as having an abnormally labile immunological apparatus. This leads to the possibility that genetic factors are more important than has been thought to be the case. Although autoimmune blood diseases occurring in man in more than one member of a family are almost unknown (Dacie, 1962), the important studies on autoimmune hemolytic anemia in NZB/BL mice (Bielchowsky, Helyer & Howie, 1959; Helyer & Howie, 1963) demonstrate clearly the potential importance of heredity (Burnet, 1963).

The occurrence of chronic autoimmune hemolytic anemia in infants is particularly remarkable (Dacie, 1962); in these cases the hypothetical abnormal antibody-forming clones seem likely to develop soon after birth and it is conceivable that the antibody-forming cells could be maternal in origin as Billingham (1959) suggested. In a recent case studied in England, acute hemolysis developed in an infant who had not thrived. Here an analogy with runt disease comes to mind. The apparent success of thymectomy in another recent case (Wilmers & Russell, 1963) suggests that the thymus gland may play a particular role in such cases, but perhaps its activities are of paramount importance in all immune processes in man in the first few months of life.

The frequent occurrence of autoimmune hemolytic anemia in cases of chronic lymphocytic leukemia and reticulosarcoma, in contrast to its rarity in other types of malignant disease, raises interesting questions. Do the malignant lymphomas represent malignancies of potential antibody-forming cells, the tumor cells of which in some instances retain their

ability to form antibodies, in particular, abnormal ones, or are the antibodies primarily formed against the abnormal neoplastic lymphoid cells which act as antigens, the antibodies at the same time having the ability to cross-react with red cells, as the work of Brody and Beizer (1963) suggests. Or do the malignant lymphomas, representing an enormous monoclonal proliferation of lymphoid cells, by occupying a great deal of the available space normally occupied by immunologically "incompetent" and competent cells, interfere with the delicate balance between the two and allow the development of a clone of autoantibody-forming cells, as has been recently suggested by Gorman and Chandler (1964)? Perhaps all these mechanisms are important.

The role of infection remains uncertain. The occurrence of acute episodes of hemolysis not too uncommonly following virus pneumonia, and rarely after other virus infections such as influenza, measles and varicella (Dacie, 1962), suggests that in certain, presumably unusually susceptible people, such infections may act as a "trigger" mechanism for autoantibody formation, but whether they do this by stimulating the formation of cross-reacting antibodies or in some other less tangible way is not clear. The former mechanism seems probable in the acute transient anemias such as follow virus pneumonia; but perhaps where autoantibody formation remains continuous, infection has in some way led to somatic mutation and to the emergence of forbidden clones of antibody-forming cells.

Finally, what significance should be attached to the recovery of a patient from an autoimmune hemolytic anemia? Clinical observation suggests that patients suffering from the cold-hemagglutinin disease, i.e., those who form high-titer cold antibodies, never cease to form the antibodies, even if followed over a 10-year period or longer. On the other hand, a small proportion of those who form warm antibodies, about 20 per cent in my experience, recover, at least temporarily, both clinically and serologically, so that their red cells ultimately cease to give a positive antiglobulin test. The latter patients seem to have been able to eliminate the abnormal clones of antibody-forming cells, if indeed these are the basis of their illness, but the former cold-antibody group never seem to be able to do this. This suggests to me a difference in etiology. This is borne out, too, by the difference in age incidence between the two groups of patients. Those suffering from the cold-hemagglutinin disease seem almost invariably to be middle aged or elderly, but the warm-antibody type of disease affects people of all ages, from infancy to old age.

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AUTOIMMUNE HEMOLYTIC DISEASE: OBSERVATIONS OF SEROLOGICAL REACTIONS AND DISEASE ACTIVITY*

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The serological characteristics of red cell antibodies in autoimmune hemolytic anemia reflect to some degree the properties and characteristics of red cell isoantibodies and may be divided by serological reactions into three main groups.[1,2]

The incomplete gamma globulin autoantibodies, which do not usually react with complement globulin and are most reactive at 37°C., are comparable to incomplete Rh antibody.

Cold agglutinins are macroglobulins which have optimum reaction at 5°C. and dissociate as the temperature is raised so that agglutination is usually abolished above 31°C.[3,4] These antibodies regularly produce complement hemolysis or cause adsorption of complement under certain conditions of temperature and *p*H.[5,6] In several characteristics cold agglutinins resemble the 19S anti-A and anti-B antibodies.

A third group of autoantibodies may be separated from others in reacting with the red cells at body temperature and binding fractions of complement globulin.[7] Agglutination of sensitized cells in antiglobulin serum is partially or wholly dependent on the adsorption of complement. The molecular species of this type of autoantibody has not been defined, but the relation to reaction with antiglobulin serum and complement resembles certain isoantibodies.

During the past decade *in vivo* observations with Cr^{51} tagged cells have demonstrated that the splenic circulation is most adapted for removing cells coated with incomplete red cell antibodies.[8-10] The rate of removal of antibody coated cells was shown to be directly related to the concentration of incomplete isoantibody used in sensitization.[8,11] There is some evidence that variations of concentration of cell bound warm incomplete autoantibody determine the activity of autoimmune hemolytic anemia.[12,13] Three additional patients are reported here in which measurements of concentration have been correlated with disease activity.

Recent studies have shown that only one antibody, the cold agglutinin, is present in this variety of hemolytic anemia and that destruction of red cells by cold agglutinins is due to the action of complement.[14,15] It has not been clear how antibodies which do not agglutinate red cells above

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31°C. can cause hemolytic anemia in the absence of chilling. In two patients with cold agglutinins and hemolytic anemia reported here, the hemolytic activity of the cold agglutinins extended to normal body temperature. In these patients complement depletion appeared to limit the severity of hemolytic anemia. Studies with I^{131} tagged cold agglutinins have extended our knowledge of the relationship of these antibodies with the red cell surface and with complement.

Methods

The methods for observing the transfer of autoantibody from patients' cells to normal cells as well as other serological procedures have been described previously.[13] Free serum antibody was titrated at one time using samples of stored serum which had been labelled as unknowns.

Radioiodination of cold agglutinin was done by the iodine monochloride method after ethanol precipitation of the serum globulin.[16] Excess iodine was removed by filtration through a resin column. Cold agglutinin was then purified by adsorption at 5°C. to whole red cells and elution at 37°C. After three such procedures the iodinated protein was relatively homogeneous in being completely adsorbed at 5°C. and released at 31°C.

I^{131} tagged cold agglutinins were incubated for one hour with whole red cells at varying temperatures after mixing at 37°C. The suspensions were washed three times in 20 volumes of fluid and the I^{131} activity was determined. Counts per 0.1 ml. of cells per minute were calculated on the basis of duplicate hematocrit determinations after incubation at 37°C.

Complement hemolysis was observed by mixing 0.25 ml. of patient's serum and 0.25 ml. of normal serum and the *p*H adjusted with HCl. Five hundredths ml. of a 50 per cent suspension of red cells was added after preincubation of all reagents at 37°C. The suspension was then incubated at the desired temperature for one hour and the percentage hemolysis determined.

Complement titers were assayed by the method described by Pillemer and coworkers.[17]

Results

(1) Relation of free serum antibody and cell bound antibody to activity of the hemolytic process during a remission induced by adrenal steroids.

A male, 67, (H. K.) had noted increasing weakness and substernal chest pain for two months without any preceding medical event. There was scleral icterus, pallor and a palpable spleen. The hematocrit was 15, Hb. 5.1 gm. per 100 cc. and red cell count was 1.23 million per cmm. with 18 per cent reticulocytes. Leukocytes were 27,000 with normal differential. Platelets were normal. Plasma bilirubin was 1.5 mg. per 100 cc. He was group A, Rh positive.

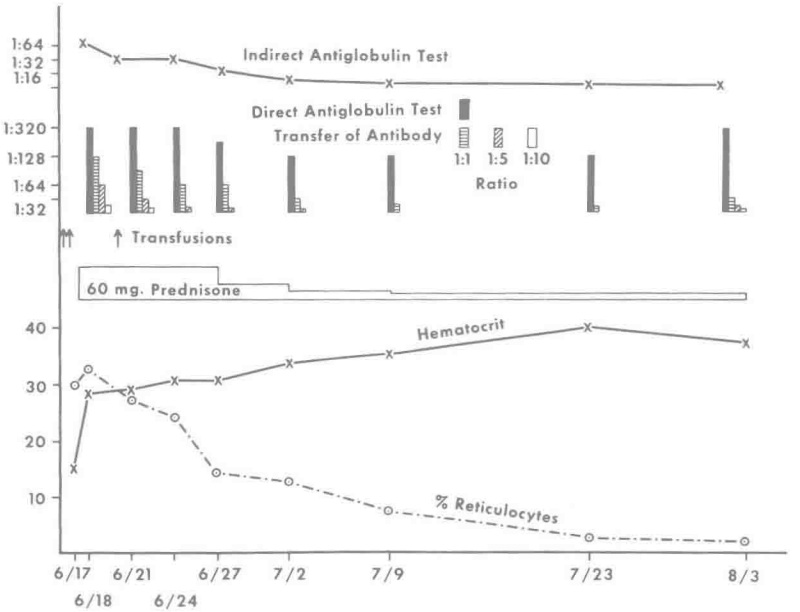

FIGURE 1. Case 1. Response of autoimmune hemolytic anemia to transfusions and prednisone. The concentration of free autoantibody was titrated in the indirect antiglobulin test. The agglutination of the patient's cells in dilutions of antiglobulin serum and the transfer of antibody from the patient's cells to various ratios of normal cells are also shown.

The direct antiglobulin test was positive to high dilution and inhibited with gamma globulin. Free serum antibody was reactive at 37°C. but was not specific for the known red cell antigens tested. The patient was given two units of group A cells in transfusion without reaction and prednisone 60 mg. daily was begun. His course showing rise in hematocrit and fall in reticulocytes is depicted in FIGURE 1. There was a fall in free serum antibody and a concomitant reduction of antibody transfer from patient's cells to normal cells. At the same time there was a return of the curve of osmotic fragility to normal. The patient has been maintained on 10 mg. of prednisone and continues to be in good health.

Comment: This active man of 67 had an onset of acute autoimmune hemolytic anemia without evidence of background infection or chronic systemic disease. Improvement in hemolytic anemia with steroid therapy closely paralleled the reduction in free serum antibody and the concentration of cell bound antibody. His response was typical of that seen in patients with incomplete warm autoantibodies.

(2) The failure of nitrogen mustard to affect the course of autoimmune hemolytic disease associated with chronic lymphatic leukemia and a subsequent response to adrenal steroids.