Chronic Lymphocytic Leukemia, Hypogammaglobulinemia and Autoimmune Hemolytic Anemia—An Experiment of Nature

By Anthony V. Pisciotta, Louis F. Jermain and Jean E. Hinz

About a third of the patients with chronic lymphocytic leukemia develop hypogammaglobulinemia at some time or other during the course of their illness. The ensuing clinical manifestations are usually no different from those encountered in hypogammaglobulinemia from other causes; the patient may manifest an "immunologic paralysis" and is thereby rendered susceptible to infections of various kinds.

The high incidence of "autoimmune" hemolytic anemia in chronic lymphocytic leukemia is also well recognized. In this complication, accelerated destruction of erythrocytes is associated with attachment of a protein to the patient's erythrocytes as demonstrated by the Coombs antiglobulin reaction.

We had the opportunity to study a patient who had the rather unusual combination of chronic lymphocytic leukemia, hypogammaglobulinemia and hemolytic anemia associated with a positive antiglobulin reaction in which the erythrocytes were shown to be coated with gamma globulin. In view of continuing evidence of "autoantibody" formation, despite hypogammaglobulinemia, our attention was directed to the manufacture of other antibodies to known antigens. In addition, information was sought relative to the "autospecificity" of the antiglobulin reaction as manifested by the possible affinity of parenterally administered labeled normal human gamma globulin for the patient's erythrocytes. The purpose of this paper is to present the results of these studies.

Methods

Hemagglutinins were sought in the serum diluted twofold serially with 0.85 per cent saline or with 20 per cent bovine albumin. Test erythrocytes were either trypsinized or untreated in 3 per cent suspension in saline or bovine albumin. The Coombs antiglobulin test was performed on thrice washed erythrocytes in 3 per cent suspension to which was added the antiglobulin serum. The reaction mixture was centrifuged at 1000 rpm for one minute and read for gross agglutination without incubation. The test battery of antiglobulin sera was produced in our laboratory from normal donors, from the sera of patients which contained autohemagglutinins of various types and from electrophoretically pure human gamma globulin. An estimate of the degree to which erythrocytes were "coated" was done by serially diluting the antiglobulin serum in saline, adding the washed test erythrocytes and noting the extent to which they were agglutinated, from 1+ to 4+, depending on the size of the agglutinates. The agglutination score is an arbitrary figure which represents a composite of the size of the agglutinates and the dilution of serum. The antiglobulin inhibition test was performed according to Dacie. Aided by a Research Grant (A-1024) from the National Institute of Arthritis and Metabolic Diseases, National Institute of Health, U.S.P.H.S., Bethesda, Md., and with the assistance of a grant from the Committee on Research, Council on Pharmacy and Chemistry, American Medical Association.
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serum was preincubated with graded serial dilutions of 4 per cent human gamma globulin in 0.85 per cent saline. The mixture was then added to a 3 per cent suspension of washed patient's erythrocytes, immediately centrifuged at 1000 rpm and read as in the Coombs antiglobulin test. The end point of this reaction was considered the first tube in which agglutination occurred. Inhibition of the antiglobulin reaction was considered to indicate that the erythrocytes were coated with gamma globulin.

The survival time of erythrocytes was performed by tagging the patient's erythrocytes with 75 μc. of chromium-51 in the form of sodium chromate. The erythrocyte radioactivity which remained in the circulation at a given time was calculated on a percentile basis, utilizing the specimen of blood 24 hours after the injection being taken as 100 per cent. No correction was made for elution of chromium. The normal survival time of 50 per cent of erythrocytes by this method is 25 to 35 days.

Electrophoresis of serum was carried out in the Spinco Model R apparatus, wherein 0.006 ml. of serum was treated electrophoretically for 16 hours, in barbital buffer, pH 8.6, ionic strength 0.05. The paper was stained with bromphenol blue and densitometric examination was accomplished on the Spinco automatic recording densitometer (Analytrol) which prepared, automatically, a graphic representation of the relative quantities of serum protein components which bound the dye.

Examination for gamma globulin was also done by the agar plate diffusion method wherein a precipitation band was sought in an agar medium which separated the patient's serum and anti-human gamma globulin rabbit serum. Gamma globulin was also sought serologically by serially diluting the patient's serum in saline in order to determine the dilution at which it inhibited the agglutination by antiglobulin serum of D-positive human erythrocytes sensitized with anti-D serum.

The life span of human gamma globulin was determined by the intravenous injection of normal human gamma globulin labeled with 50 μc. The preparation contained 3.75 mg. gamma globulin per 30 ml. with a specific activity of 122 μc. of 131I per milligram. Chromatographic analysis showed 98.3 per cent human gamma globulin and 1.7 per cent iodine. Blood was removed every 30 minutes for two hours, then every 24 hours. The erythrocytes were separated from the plasma, washed 3 times with saline; 1 ml. of packed erythrocytes was hemolyzed with 2.0 ml. distilled water. Two ml. of the patient's plasma were placed in clean test tubes, and all specimens were counted on the same day in a well-type scintillation counter. Radioactivity of the surviving 131I gamma globulin was expressed in counts per minute (CPM) per milliliter of plasma or of washed, packed erythrocytes.

CLINICAL DATA

A 75 year old white man developed lobar pneumonia in February, 1956. He was treated with antibiotics, and the manifestations of pneumonia subsided. Several blood transfusions were given because of anemia. During the next six months, he continued to have marked weakness and dyspnea, and he lost 25 pounds. The demonstration of a large spleen, pallor, leukocytosis with lymphocytosis and anemia led to a course of irradiation to the neck and 6 transfusions, with little benefit. In May, 1957, he was admitted to Milwaukee County Hospital because of substernal pain, dyspnea, weakness and a cough productive of whitish sputum. The positive physical findings included pallor and evidence of great weight loss. The sclerae were icteric. The cervical lymph nodes were less than 1 cm. in diameter, firm and nontender. There was dullness at both lung bases, with fine crepitant rales. The spleen was palpable 17 cm. and the liver 10 cm. below their respective costal margins.

The initial blood study showed the following values: hemoglobin 3.0 Gm./100 ml.; RBC, 950,000/cu.mm.; WBC, 168,500/cu.mm.; band forms, 1 per cent; segmented neutrophils, 5 per cent; lymphocytes, 93 per cent; N. myelocytes, 1 per cent; platelets, 98,000/cu.mm.; reticulocytes, 1.4 per cent and sedimentation rate, 167 mm. in one hour. The bone

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narrow was packed solidly with small lymphocytes, which seemed mature in appearance. A few normoblasts were present.

The total serum bilirubin was 3.0 mg. 100 ml., mostly indirect in reaction. The fecal urobilinogen excretion in 24 hours was 1210 mg. Osmotic fragility showed beginning hemolysis at 0.44 per cent saline and complete hemolysis at 0.3 per cent saline. The mechanical fragility showed 15.4 per cent liberation of hemoglobin after 90 minutes of shaking. The Coombs' antiglobulin test was 3+ positive. Examination of the serum for hemagglutinins showed agglutination of the patient's own trypsinized erythrocytes at 3 C. in a dilution of 1 to 32 of the patient's serum in 0.85 per cent saline. At 37 C. hemagglutinins were not demonstrated utilizing untreated erythrocytes in saline or in bovine albumin. The Donath-Landsteiner test was negative, and hemolysins were not demonstrable.

The survival time of 50 per cent of the patient's erythrocytes, labeled with chromium-51, was established at 10 days. During this time, there was significant sequestration of the red cells in the spleen as evidenced by radioactivity counts over the surface area of the spleen which exceeded those over the precordial area by more than double.

By chemical determination, the total serum protein was 5 Gm. 100 ml., of which 4 Gm. was albumin and 1.0 Gm. was globulin. Electrophoresis of serum showed a greatly diminished gamma globulin band (fig. 1). Densitometric examination of the paper strip on the analytrol apparatus showed the following values: albumin, 70.2 per cent; alpha-1 globulin, 3.8 per cent; alpha-2 globulin, 10.2 per cent; beta globulin, 9.9 per cent; and gamma globulin 5.4 per cent.

The diagnosis of chronic lymphocytic leukemia, autoimmune hemolytic anemia and hypogammaglobulinemia was established. The pertinent data obtained during the course of this patient's illness are illustrated in figure 2. Adrenocorticotropic hormone (ACTH) gel was given intramuscularly, 100 U. daily. In 10 days, a reticulocyte peak of 29 per cent occurred. On the eleventh treatment day, the patient received an intramuscular injection of 20 ml. of human gamma globulin which was repeated at intervals indicated in figure 2. On the fifteenth day, administration of ACTH was discontinued and replaced with prednisone, 60 mg. daily, gradually reduced to a subsequent maintenance dosage indicated in figure 2. The erythrocyte and hemoglobin values increased to $3.5 \times 10^6$ and 10 Gm., respectively, by the fiftieth day, and the patient gained in comfort and in well being. The

![Fig. 1.—Electrophoretic pattern of patient's serum.](image-url)
Coombs' antiglobulin reaction remained strongly positive throughout the course of the patient's illness. By electrophoretic estimation, the gamma globulin remained in the vicinity of 5 to 6 per cent of serum proteins for the entire period of observation. Serologic study confirmed the continuing presence of gamma globulin, as the patient's serum diluted 1:1024 inhibited the antiglobulin reaction of D-positive normal erythrocytes sensitized with anti-D serum. On the eighty-fifth day, there was a marked and striking decrease in antiglobulin inhibitory activity of the patient's serum to 1:64.

At this time, he developed fever, malaise, pain in the right side of the chest and cough productive of purulent sputum. Signs of consolidation and course rales were present bilaterally. A friction rub was heard on the right. The patient failed to make improvement when penicillin and gamma globulin were given. He expired on the ninetieth day of observation. An autopsy was not performed.

**Special Studies**

The reaction of the patient's erythrocytes with our battery of antiglobulin sera is shown in figure 3. The initials along the abscissa signify patients with autoimmune hemolytic disease from whose sera the rabbit anti-human globulin sera were prepared. The white bar is a serum prepared from a cold-agglu-
Fig. 3.—Titers of battery of antiglobulin serums against patient's erythrocytes. The test erythrocytes are coated with a warm incomplete antibody, derived from gamma globulin.

tinin-containing serum. The four black bars were antiglobulin serums prepared from patients with a "warm antibody" type of hemolytic anemia. The stippled bar represents the antiglobulin titer to a serum prepared from pure gamma globulin. The striped bar is antiglobulin serum prepared from a normal man.

It will be seen that the cells were strongly agglutinated by all antiglobulin serums, including the one prepared from human gamma globulin. This affirmed directly that the protein which coated the patient's erythrocytes was at least partly derived from gamma globulin. Highest titration scores were observed with 2 antiglobulin sera prepared from human sera which contained a "warm" hemagglutinin. Preincubation of the Coombs antiglobulin serums with serial dilutions of human gamma globulin inhibited the antiglobulin reaction to a dilution of 1 to 4096 of the gamma globulin (table 1). This

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<th>Dilution of 4% normal human gamma globulin</th>
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affirmed that the protein which coated the patient’s erythrocytes was gamma globulin alone and not a mixture of all serum proteins.

_Serum protein studies._—The patient’s blood type was A, but anti-B agglutinins were present in his serum in a dilution of 1 to 2. The purified protein derivative (P.P.D.) tuberculin skin test was negative. There were no typhoid or paratyphoid agglutinins demonstrable in the patient’s serum. None had appeared 4 and 8 weeks after the patient was challenged with triple typhoid vaccine.

The findings of hypogammaglobulinemia were confirmed by other methods. An agar-gel diffusion plate was prepared to demonstrate a reaction between the patient’s serum and anti-human globulin rabbit serum. A line or precipitate appeared in the interface between these two substances, showing that gamma globulin was actually present though in diminished amount. The patient’s serum, diluted 1 to 32, inhibited agglutination of anti-D sensitized D+ erythrocytes by antiglobulin serum (table 2). These studies show evidence of diminished amounts of gamma globulin as well as impaired immunologic function.

_Gamma globulin survival studies (fig. 4)._—The half-life of the $^{131}I$ gamma globulin was observed to be six days in the patient, taking the 24 hour reading as 100 per cent. This value falls in the normal range established in our laboratory with this technic.

During this period of observation, the patient’s washed, packed erythrocytes were examined for increase of radioactivity which might indicate possible adherence of the radioactive gamma globulin to the surface of the patient’s circulating red cells. The erythrocytes had been labeled with chromium-51 one month before to determine their survival time. Before the $^{131}I$-gamma globulin was given, there was sufficient residual radioactivity in the washed, packed erythrocytes to give a background count of 400 counts per minute per 1 ml. packed RBC attributed to chromium-51. One hour after the injection of $^{131}I$-gamma globulin, the radioactivity of the erythrocytes rose to 840 CPM/1 ml. packed RBC, while that of the plasma was 37,700 CPM/1 ml. plasma. Two hours later, the radioactive counts of the RBC fell to the baseline value and then erythrocyte radioactivity declined in a manner expected of disappearance of red cells labeled with residual

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Fig. 4.—Survival time of $^{131}$I normal human gamma globulin in patient’s circulation.

chromium-51. During this period of observation, no additional radioactivity appeared on the patient’s erythrocytes. It may, therefore, be stated that there was no affinity of gamma globulin from normals for the patient’s erythrocytes.

**Discussion**

It seems clear that the absence or diminution of gamma globulin is not necessarily correlated with complete loss of immunologic function. Chaplin,17 and Painter and Korst18 have shown that incompatible erythrocytes, labeled with chromium-51, disappear from the circulation promptly in patients with agammaglobulinemia. Patients with agammaglobulinemia have been described to develop diseases of allergo-immunologic origin.19-21 Several cases of bronchial asthma18,19 have been reported in patients with agammaglobulinemia. A peculiar sequence of events was described by Janeway20 in his patients with congenital agammaglobulinemia who developed foreign protein reaction resembling lupus erythematosus when given repeated doses of gamma globulin. Rheumatoid arthritis is known to occur simultaneously with agammaglobulinemia.21 The in vivo occurrence of immunologic reactions does not necessarily presuppose circulating gamma globulin or indeed serologic evidence of antibody in vitro. In autoimmune hemolytic disease, demonstration of hemagglutinins in the serum in vitro is an unusual finding,22 and in many cases, antibody can be demonstrated only by examin-
ing the erythrocytes by the antiglobulin test. It is evident that such erythrocytes must have been sensitized in vivo even though a "sensitizing protein" is not demonstrable in serum in vitro.

There is a difference of opinion regarding the mechanism of attachment of serum protein to the red cell. The antiglobulin test, though in itself a nonspecific reaction, is popularly considered to result from an autoimmune reaction wherein the patient produces an antibody which attacks all erythrocytes, including his own. On the other hand, there is reluctance to accept this concept of autoimmunization because the identity of a possible auto-antigen which could have stimulated the formation of autoantibody is unknown. Also, the mechanism of manufacture of antibodies against one's own proteins have never been satisfactorily explained. Jandl has shown that a positive antiglobulin reaction may be produced by means other than immunization. Treatment of red cells by heavy metals, silicic acid, etc., may result in injury to the erythrocyte which may then be followed by (protective?) coating of the red cell by serum protein, as demonstrated by the antiglobulin reaction. Unpublished studies from our laboratory disclose that all serum fractions take part in this type of reaction and that this mechanism for attachment of serum protein presupposes a damaged erythrocyte. On the other hand, the erythrocyte-coating protein in our patient involved the gamma globulin fraction alone. Because normal gamma globulin did not attach to the patient's erythrocytes, it is probable that this protein must have been derived from a source endogenous to the patient.

The factors which underly an abnormally low gamma globulin value are not completely understood. The principal evidence points to a defect in the synthesis of gamma globulin. Knowledge of the vivo manufacture of this protein is indirectly acquired through a consideration of the life span of gamma globulin. Unfortunately, the scope of this information is limited because of the lack of a satisfactory methodology for the measurement of the life span of a person's own gamma globulin in his own circulation. Two methods are presently available whereby one may measure approximate survival time of this protein in a foreign circulation. The first is based upon the disappearance of radioactivity from the circulation of a person given gamma globulin labeled with a radioactive isotope such as iodine-131 or sulfur-35. This method consistently gives results much lower than the life span of human gamma globulin based on immunologic technics. The second method involves the intravenous infusion of a gamma globulin rich in easily quantitated and identifiable antibodies. The disappearance time of antibody is considered to be the same as survival of gamma globulin. When gamma globulin which contains a variety of antibodies is given, there is a pronounced variation in survival time of different antibody proteins, which affirms the heterogeneous nature of the proteins which make up gamma globulin.

The normal value for half life of \(^{131}\text{I}\)-labeled gamma globulin in our patient suggests two possible interpretations. The popular view is that hypogammaglobulinemia is due to a failure of synthesis of gamma globulin. It is also
possible that gamma globulin was produced in normal amount, only to be-
come attached to his erythrocytes and then carried off to become destroyed
at the same rate as the red cells. This remote possibility hardly seems likely.

SUMMARY AND CONCLUSIONS

1. The case study is presented of a 75 year old man who had chronic
lymphatic leukemia, autoimmune hemolytic anemia and hypogammaglobu-
linemia. The positive antiglobulin reaction with serum made from gamma
globulin and the neutralization of the antiglobulin reaction with human
gamma globulin demonstrated that this patient’s erythrocytes were coated
with gamma globulin.

2. There was a normal survival time of $^{131}$I-labeled normal human gamma
globulin, suggesting defective synthesis of gamma globulin. Failure to
demonstrate radioactivity on the patient’s erythrocytes when $^{131}$I-labeled nor-
mal gamma globulin was given signified that normal human gamma globulin
has no affinity in vivo for the patient’s red cells and that the erythrocyte-coating
protein was derived from a source endogenous to the patient.

3. These relationships favor an immunologic mechanism in the development
of an antiglobulin reaction in this patient.

SUMMARIO IN INTERLINGUA

1. Es presentate le studio del caso de un masculo de 75 annos de etate qui
habeva chronic leucemia lymphocytic, anemia hemolytic autoimmun, e hypog-
ammaglobulinemia. Le positivitate del reaction antiglobulinic con sero facite
ex globulina gamma e le neutralisation del reaction antiglobulinic effectuate
per globulina gamma human demonstrava que le erythrocytos del patiente
esseva revestite de globulina gamma.

2. Esseva constatate un normal longevitate de normal globulina gamma
human (marcate con $^{131}$I), un facto que pareva indicar le presentia de un
defecto in le synthese de globulina gamma. Post le administration de normal
globulina gamma con marcation per $^{131}$I, le erythrocytos del patiente revelava
nulle radioactivitate. Isto indicava que normal globulina gamma human ha
nulle affinitate in vivo pro le erythrocytos del patiente e que le proteina re-
vestiente le erythrocytos del patiente es de crigine endogene.

3. Iste considerationes argue in favcr de un mechanismo immunologic in
le disveloppamento del reaction antiglobulinic in le caso del presente patiente.

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