EXPERIMENTAL PRODUCTION OF CIRCULATING ANTIBODIES TO CHROMIUM

HAIM A. COHEN, M.D.

A positive potassium bichromate patch test in cases of contact type eczema due to cement, shoe leather, or other materials, suggests sensitivity to chromium to be an underlying factor (1-8). It is known that inorganic salts of metals may act as allergens, but no circulating antibodies to such inorganic compounds have yet been detected. Some simple organic compounds, however, when injected in the form of conjugated antigens, are capable of stimulating circulating antibody production in animals (9).

Keogh, North and Warburton (10) found that an extract of hemophilus influenza can be attached to red blood cells and renders them specifically agglutinable by homologous bacterial antibodies. They later reported similar findings with several other polysaccharide antigens of bacterial origin (11). This type of reaction has been referred to as indirect, passive, or conditional hemagglutination (12). Boyden (13) observed that washed sheep erythrocytes treated with dilute solutions of tannic acid can absorb protein, and that they are then agglutinable by specific antisera to the attached protein. These observations have since been confirmed and extended (12). Landsteiner and van der Sheer (14) linked diazo compounds to red blood cell “ghosts”. The complex became agglutinable by specific antisera to azoproteins derived from these compounds. Pressman, Campbell and Pauling (15) showed that sheep erythrocytes to which azophenylarsonate or ovalbumin had been attached were agglutinable by the specific antisera.

It therefore seems that erythrocytes, after sensitization with polysaccharides, proteins, or simple organic compounds, can both induce antibody production to the attached substance, and react in vitro with specific antisera to attached compounds, and produce hemagglutination.

Before describing the experiments based on this speculation, the phenomenon of the direct agglutination of erythrocytes by metallic cations merits consideration. Jandl and Simmons (16) thought such agglutination to result from firm attachment of the metallic cations (e.g. chromium) to the red cell membranes. The consequent reduction of the negative surface charge might then permit erythrocyte aggregation. They also thought it possible that the metallic cations might form direct cross-linkages between the red cells. The hemagglutination was found to be inhibited by substances containing polycarboxylic groups, and by normal human or animal serum. The serum may have acted either by competing with the erythrocytes for the binding of the available chromium, or by providing a protective coating around the chromium-red cell complex.

In the experiments described in this paper, the metallic ion was joined to the surface of human erythrocytes, and the conjugated antigen was then injected into rabbits in an attempt to stimulate the production of antiserum containing specific antibodies to chromium. It was thought possible that such an antiserum, added to the chromium-red cell complex in vitro, might produce an agglutination phenomenon instead of a protective one.

MATERIALS AND METHODS

Preparation of the antigens for immunization. 2.0 ml of packed group “O” human erythrocytes were added to 0.2 ml-0.4 ml of a 0.5% potassium bichromate solution (0.03 M sol) in 10 ml of normal saline. After standing at room temperature for 15 minutes to allow the ions to become attached to the erythrocyte surface, the red cells were thrice washed with normal saline and re-suspended in another 10 ml of normal saline, giving a 20% suspension. A similar procedure was followed for the preparation of chromium chloride-sensitized erythrocytes.

Immunization of the rabbits. Rabbits of 2 kg–3

* From the Department of Dermatology and Venereology, Hadassah University Hospital, Jerusalem, Israel. This work was aided in part by grants from the Frances Pascher Research Fund in Dermatology, the Cantor Fund, and the Estate of Nellie Neuhau sen.

Received for publication April 1, 1961.
kg. weight were used, and were separated into four groups.

**Group I:** Five rabbits were each given six bi-weekly intravenous injections of 2.0 ml of the potassium bichromate-sensitized erythrocyte suspension.

**Group II:** Seven rabbits were similarly treated with the chromium chloride-sensitized erythrocytes.

**Group III:** Two rabbits were each given two intramuscular injections, at 15 day intervals, of 0.5 ml of Freund's adjuvants (17) containing potassium bichromate.

**Group IV:** Two rabbits were similarly treated with 0.5 ml of Freund's adjuvants containing chrome chloride and crystalline bovine albumin.

**Preparation of sensitized erythrocytes for in vitro test.** 0.2 ml of fresh, packed and washed group "0" human erythrocytes from defibrinated blood, were suspended in 10 ml of normal saline containing 0.1 of the stock solution of 0.5% potassium bichromate or chromium chloride. After standing for 15 minutes at room temperature the red cells were thrice washed in normal saline and resuspended in another 10 ml normal saline. After initial centrifugation at low speed for 2 minutes, the red cells sensitized with chromium chloride were found to be strongly agglutinated at the bottom of the test tube. This did not occur at all with the potassium bichromate-sensitized cells. Gently shaking the agglutinate each time in fresh normal saline broke the mass down to small agglutinates of varying sizes. After the third washing a fine suspension of very small agglutinates was obtained.

**Performance of the in vitro test.** The antisera were first inactivated at 56° C for 3 hr., and the agglutinins against erythrocytes were removed by repeated absorptions at room temperature. The test procedure follows:

1. Prepare serial dilutions of 0.2 ml antisera and of control sera, in 0.2 ml normal saline.
2. To each dilution add 0.1 ml of 2% sensitized red cell suspension, and shake.
3. Allow the erythrocytes to sediment at room temperature.

**Controls.** Sera taken from rabbits before immunization and sera from non-immunized rabbits were similarly treated and used as control sera. In addition, the antisera were also tested with non-sensitized erythrocytes.

**Reading of the results.** The results were read several times from 2 to 24 hours after the performance of the test. The form of sedimentation of the red cells as seen through the bottom of the test tube was evaluated according to the criteria of Salk (18). A negative result was evidenced by a sediment in the form of a small, compact, button-like mass. A positive result, representing passive hemagglutination, consisted of sedimentation spread over a much wider area. Blurring or raggedness of the edges of this area indicated a high antibody titer in the serum.

**RESULTS**

Three rabbits died during the period of immunization, two from Group I and one from Group II. The remaining rabbits died from unascertained causes within 3 weeks of the end of immunization.

The sera of the Group I rabbits, which were immunized with potassium bichromate-sensitized red cells, did not contain antibodies against potassium bichromate-sensitized erythrocytes, but contained antibodies against chromium chloride-sensitized erythrocytes (Fig. 1).

The sera of Group II rabbits contained antibodies against chromium chloride-sensitized erythrocytes (Fig. 1).

The sera of Group III and Group IV rabbits did not contain antibodies against either potassium bichromate or chromium chloride-sensitized erythrocytes.

**INVESTIGATION OF SPECIFICITY**

The following experiments were performed to determine the specificity of the antibodies to chromium.

**Experiment I: Substitution of Sheep for Human Erythrocytes**

To determine whether the specificity of the antibodies was independent of the particular red cell used, experiments using sensitized sheep erythrocytes were performed. The inactivated rabbit antisera used were first treated with packed, normal sheep erythrocytes to eliminate heterophil antibodies against sheep red cells. The cells tested were sensitized by the same procedure as that used for the human cells. All the sera tested gave positive results, suggesting that the reaction was not dependent upon the erythrocyte used.

**Experiment II: Substitution of Erythrocytes Sensitized with Other Metals**

To determine whether the antibodies were specific only to chromium, the test was repeated with human group "0" erythrocytes sensitized with FeCl₃, AlCl₃, NiCl₂, and CoCl₂. Because
FIG. 1. Reactions of rabbits injected with either potassium chromate sensitized erythrocytes or chromium chloride sensitized erythrocytes tested against chromium chloride sensitized erythrocytes. Rows A–E represent individual immunized rabbits. Row F is a normal rabbit prior to immunization. Columns 1–5 represent progressive serial dilutions of the immune sera (dilutions 1:4 to 1:64). Column 6 represents undiluted immune sera tested against normal (non-sensitized) erythrocytes.

In all except the control tubes there is hemagglutination, indicating the presence of antibodies to chromium chloride. (The blank spaces represent tubes removed because of hemolysis.)

ferric chloride and aluminum chloride themselves strongly agglutinate red cells, sensitization with these salts was done with dilutions of 0.01 ml and 0.025 ml respectively, of a 0.5% stock solution, per 0.2 ml packed erythrocytes. A parallel control was carried out, using red cells sensitized with chromium chloride in a dilution of 0.025 ml of an 0.5% stock solution of 0.2 ml packed red cells. All the reactions were negative with the exception of the control and that in which ferric chloride-sensitized red cells were used.

**Experiment III: Previous Absorption of Antibodies to Chromium by Packed Sensitized Erythrocytes**

Antisera from Group I and Group II rabbits were inactivated and absorbed with normal group "O" human erythrocytes. The sera were then tested in a dilution of 1:2, against the suspension of chromium chloride-sensitized erythrocytes. The test was then repeated with the same sera after they had undergone one to three successive absorptions with packed, sensitized to chromium
chloride erythrocytes. The results (Fig. 2) seemed to indicate that antibodies to chromium had been absorbed by the packed, sensitized erythrocytes, and that the previously positive sera had become negative.

Experiment IV: Demonstration of Inhibition Reaction

If an antigen is added to homologous antiserum before the sensitized erythrocytes are introduced, specific antibodies should combine with the antigen and the positivity of the hemagglutination reaction may be diminished or abolished. When metallic cations are used as antigens some complicating factors are introduced. These are discussed later.

(a) 0.1 ml of a 2% suspension of chromium chloride-sensitized sheep erythrocytes was added to each of five tubes containing 0.2 ml of antiserum in dilutions of $\frac{1}{2}-\frac{1}{52}$ (Fig. 3, row A)
and to five tubes containing 0.2 ml of normal rabbit serum in similar dilutions (Fig. 3, row B). Heterophil antibodies had been removed from the sera by previous absorptions. After standing at room temperature for 10 minutes, 0.2 ml of a 1/1000 dilution of the 0.5% stock solution of chromium chloride was added to each tube. All of the five tubes containing antiserum showed a definite positive hemagglutination, indicating that the subsequently added chromium chloride was unable to inhibit the hemagglutination reaction. Of the five tubes containing normal rabbit serum, the first two tubes showed no hemagglutination. A slight hemagglutination occurred in the third tube and a definitely positive hemagglutination in the fourth and fifth tubes.

(b) The experiment was repeated using the same materials and quantities, but in this case the chromium chloride was added 10 minutes before introducing the sensitized red cells. In the tubes containing antiserum (Fig. 3, row C) inhibition of the reaction occurred in the first tube, whilst in the remaining four tubes there was positive agglutination. Of the tubes containing normal serum (Fig. 3, row B), the first three showed no hemagglutination, but there was a positive hemagglutination in the fourth and fifth tubes.

A possible interpretation of some unexpected results in these inhibition experiments is given in the discussion.

**DISCUSSION**

The passive hemagglutination reaction, which utilizes erythrocytes as carriers of antigenic material, has frequently been used in the search for specific circulating antibodies to polysaccharides and proteins (19-26). The antigen in the present experiments was a metallic cation; it attaches to the erythrocyte directly as when a polysaccharide antigen is used. Metallic cations are capable of producing smaller or greater degrees of direct hemagglutination, varying with the concentration of the metallic ions. Jandl and Simons (16) suggested that metallic cations are bound to the erythrocyte surface by COOH groups. The inhibition of this direct agglutination by certain organic materials was apparently due to the fact that the latter also possessed this carboxyl grouping. The most strongly negatively charged serum constituents will most strongly attract the metallic cations, union being most rapid with albumin, followed in descending order by α, β, and γ globulins and fibrinogen. The fine agglutinates present in these tests, in which low concentrations of metallic cations were used, did not interfere with the phenomenon of passive hemagglutination occurring after the addition of antibody.

The results of the experiments reported in this paper suggested that the sera of the immunized rabbits contained specific antibodies to trivalent chromium. Thus, the passive hemagglutination test was positive with chromium chloride-sensitized sheep, as well as human erythrocytes, and was negative in most cases in which other metals were substituted for the chromium. The exception in the case of iron might have been due to the production of antibodies to this metal during the immunization process, in which there was a liberation of hemoglobin from the sensitized human erythrocytes injected into the rabbits. Further evidence for the presence of specific antibodies was the progressive weakening and final abolition of the hemagglutination reaction following treatment of the antiserum with packed chromium chloride-sensitized red cells.

Interpretation of the inhibition reaction experiments was somewhat less simple. It must be noted that metallic cations were used as antigen. Polysaccharide or protein antigens will only attach themselves specifically to antibodies, but metallic cations may, in addition, become non-specifically attached to any or all of the protein fractions of the serum. In experiments 4(a) and 4(b) the hemagglutination which occurred in the tubes containing the two or three highest dilutions of normal rabbit serum (Fig. 3, rows B and D) was unexpected. It is possible that the excess of chromium cations, resulting from the higher dilution of the serum, was in some way capable of forming complexes between the normal serum proteins and the sensitized erythrocytes, producing a non-specific hemagglutination. The hemagglutination which occurred in all except the lowest of the dilutions of antiserum to which chromium chloride had been added prior to adding erythrocytes (Fig. 3, row C), could be due to the same mechanism. However, a comparison of the second and third dilutions in this series with the equivalent dilutions in the control, normal serum (which showed no hemagglutination) indicates that at these
dilutions there may be a greater affinity between the immune serum proteins, the excess of metallic eations, and the sensitized red cells than exists in the case of normal serum. (Fig. 3, rows C and D, tubes 2, 3).

The fact that the sera of the rabbits given injections of potassium bichromate-sensitized erythrocytes did not contain antibodies to potassium bichromate (hexavalent chromium) but only to chromium chloride (trivalent chromium), is of interest from both experimental and clinical viewpoints. By contrast, in patients with chromium dermatitis the patch test to potassium bichromate is positive and that to chromium chloride negative. An explanation of this apparent paradox may lie in the fact that polysaccharides are known to be capable of reducing the hexavalent chromium of chromate salts to trivalent chromium. The skin, with the exception of the stratum corneum, is rich in polysaccharides, which may thus act as reducing agents for chromates penetrating into or through the ground substance. Penetration of chromium through the skin was studied in 6 normal and in 6 chromium-sensitive individuals (27). Within 24 hours of the application of radioactive chromate to the skin, radioactivity was detected in erythrocytes, plasma and urine. There was no quantitative difference between the two groups tested. Individuals suffering from contact dermatitis due to chromium have probably absorbed large amounts of this substance, mainly through their skin, over considerable periods of time. The chromium may have entered the circulation either as a chromate or as a trivalent chromium salt after reduction by polysaccharides of the ground substance. Even in the former case, however, it seems probable that the chromate would be finally reduced to trivalent form. Parallel phenomena have been observed with azodyes and other chemical substances, both in animals and in sensitized individuals (28–33).

Assuming that reduction of chromate to trivalent chromium does take place in the skin of humans, the results of the patch tests to potassium bichromate and to chromium chloride in chromium-sensitive patients might be explainable as follows: The externally applied chromate penetrates the stratum corneum, without forming any complexes with the negatively charged proteins or other substances present in this layer. At a deeper layer it undergoes reduction. The resultant trivalent chromium reacts or combines with the polysaccharides of the ground substance, or with protein of a different nature from those present in the stratum corneum. To this complex antibodies are formed. Externally applied trivalent chromium, by contrast, readily combines with the negatively charged albuminoid-type proteins in the stratum corneum. To such a complex the individual suffering from "chromate" dermatitis has formed no antibodies, and the chromium chloride patch test result is therefore negative.

The experiments here described may compliment those of Frei (34), Sulzberger (35), and Haxthausen (36–37), who induced hypersensitivity to metals in animals and in human. The last author gave intradermal injections of mercury, chronic acid, or formalin, mixed with horse serum, to individuals previously demonstrating negative results to patch tests with these substances. In each case sensitivity to the substance injected was produced. Attempts to demonstrate the presence of reagins by the Prausnitz-Kustner test were unsuccessful. Landsteiner and Chase (38) induced skin sensitivity in guinea-pigs by the intraperitoneal injection of simple chemical compounds and homologous red cell ghosts.

Under certain experimental conditions in guinea-pigs circulating antibodies to chemical compounds has been produced, depending upon the nature and amount of the antigen injected (39–42). The present work indicates that a somewhat similar phenomenon can be demonstrated in rabbits.

**SUMMARY**

It was demonstrated, by means of the passive hemagglutination reaction, that the sera of rabbits given intravenous injections of human Group “O” erythrocytes sensitized with either potassium bichromate or chromium chloride contained antibodies only to the trivalent chromium. The sera of rabbits injected with the same chromium salts plus Freund adjuvants and Crystalline bovine albumin did not contain antibodies to chromium.

**ACKNOWLEDGMENT**

Grateful thanks are expressed to Professor A. L. Oltzki, Head of the Bacteriological Laboratory, Hebrew University-Hadassah Medical School, for helpful advise.
REFERENCES


27. Unpublished Data.


