Experimental Production of Cold Agglutinins in Rabbits

By Nicolas Costea, Vincent Yakulis and Paul Heller

HEMOLYTIC ANEMIA of the cold agglutinin (CA) variety is believed to represent an autoimmune disorder occasionally complicating the course of lymphoproliferative diseases.1,2 Whereas animal models have been proposed for the investigation of various autoimmune phenomena,3 cold reacting erythrocyte autoantibodies have not yet been studied in experimental animals. It is the purpose of this report to present studies of the kinetics, serologic and immunochemical characteristics of erythrocyte antibodies of the cold reacting variety which were induced in rabbits by immunization with a xenogeneic antigen.† These studies were stimulated by a previous observation that rabbits produced cold hemagglutinins when injected with heat killed Listeria monocytogenes (HKLM) isolated from a patient with Listeria monocytogenes septicemia and cold agglutinin hemolytic anemia.5

Materials and Methods

Listeria monocytogenes of five different serotypes were cultured on brain-heart-infusion medium at room temperature for 24 hours. The bacterial suspension was centrifuged and washed three times in a large excess of sterile saline. The final suspension was adjusted to contain 10⁸ microorganisms per ml. as measured by nephelometry (McFarland’s standards). The bacterial suspension was heated for 1 hour at 70 C. The sterility of the HKLM suspension was ascertained by routine bacteriological methods. L. monocytogenes was also grown on synthetic folic acid assay medium (Difco B318) to which folic acid, 0.2 mg. per liter, was added. The antigenicity of the microorganisms grown on organic or synthetic media was identical.

Immunization procedure: Seventy rabbits, either male or female, weighing 2.5 to 4 Kg. were injected intravenously with 6 x 10⁸ HKLM serotype 1, 2, 3, 4a or 4b, in three divided doses at intervals of 1, 4, or 24 hours. Blood samples were collected from the orbital sinus through capillary glass tubes and allowed to clot at 37 C. The serum was separated by centrifugation in a warm centrifuge and if not immediately used was stored at −20 C. without preservative. Small groups of mice, rats, guinea pigs and dogs were also injected intravenously with HKLM serotype 4b.

Antibody assay: The heat labile fractions of complement were inactivated by heating

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†Terminology suggested by Gorer et al.4
the sera at 56 C. for 30 minutes. Cold agglutinins (CA) were titrated by serial twofold dilutions of 0.2 ml. of serum in saline. Clean pipets were used for each dilution step to avoid carry over. Volumes of 0.05 ml. of a thrice washed 2 per cent erythrocyte suspension were added to each tube and incubated at 4 C. for 2 hours. The agglutination end point was determined macroscopically after centrifugation at 4 C. for 15 seconds in an Adams Serofuge. The erythrocytes used in these experiments were autologous, allogeneic (adult and cord blood) or xenogeneic. The latter were obtained from human adult or cord blood. The adult cells were either I positive, I negative or PNH erythrocytes. Other xenogeneic erythrocytes were obtained from guinea pigs, dogs, cats, mice and oxen. When multiple erythrocyte samples were quantitatively tested for their agglutinability by one particular serum, serially diluted serum samples were set up in a "bulk titration" system and the erythrocytes were then added. Titters were recorded as the reciprocal of the highest final dilution of serum showing macroscopically definite erythrocyte agglutination. The bacterial antibodies were estimated by incubating the serially diluted sera at room temperature for 3 minutes on rotating glass plates with 0.05 ml. of HKLM suspension containing 10⁰ microorganisms per ml. Agglutination was observed macroscopically. No significant amounts of HKLM antibodies could be detected in the rabbits prior to immunization.

Rabbit sera were subjected to starch block electrophoresis according to the method of Kunkel and Slater. Starch block sections were eluted with 0.15 N borate buffer pH 8.0 and the eluates were concentrated by vacuum dialysis. The relative protein concentration was determined by measurement of the O.D. at 280 m in a Beckman Model D.U. spectrophotometer.

Six ml. of heat inactivated rabbit serum were equilibrated by dialysis with 0.15 N phosphate buffer pH 8.0 and applied to a Sephadex G 200 column 150 cm. in height and 1 cm. in diameter. Elution was carried out with the same buffer at room temperature under a hydrostatic pressure of 5 cm. Fractions of 2 ml. volumes were collected and the protein and antibody content determined in each fraction.

Inhibition of serum γM-antibodies* by mercaptans: Rabbit sera or purified protein fractions were incubated at 37 C. for 1 hour with equal volumes of 0.2M 2-mercaptoethanol or 0.2 d, l-penicillamine in phosphate buffered saline (pH 7.3). Complete inactivation of rabbit γM-antibodies was achieved by this treatment while γG-antibodies were not affected.

Preparation of I¹²⁵-labeled cold agglutinin: The protein solutions representing the first chromatographic peak were pooled, concentrated by vacuum dialysis and labeled with I¹²⁵ by the iodine monochloride exchange technique of Helmkamp et al. Amounts of iodine monochloride and carrier free I¹²⁵ calculated to yield 0.5 atoms of iodine per molecule of protein (assumed molecular weight 10⁶) were added. Human or bovine serum albumin or fresh rabbit serum was added to a final concentration of 6 per cent to reduce self-radiation and monolayering on glass surfaces of the labeled protein. Free iodine was removed by dialysis against saline. After dialysis 98 per cent of the total radioactivity was protein bound as demonstrated by precipitation with 10 per cent trichloracetic acid. The labeled macroglobulin solution was diluted in 5 volumes of buffered saline, pH 7.3 and incubated with 3 volumes of washed, autologous rabbit erythrocytes at 4 C. for 2 hours. The strongly agglutinated erythrocytes were then washed 6 times in large volumes of chilled buffered saline. After the last washing the agglutinates were incubated at 38 C. for 30 minutes. The supernatant containing the CA was collected by centrifugation at 38 C. for 10 minutes (500 g). The association and dissociation cycle was repeated once to insure purity of the final antibody suspension. The eluate was concentrated by vacuum dialysis and subjected to immunoelectrophoresis. The labeled γM isolated by thermic elution had a CA titer of 256 at 4 C. while the original serum had a titer of 1024.

*Nomenclature suggested by the WHO committee for human immunoglobulins.⁷
¹Na I¹²⁵, carrier free, without reducing agents, Iso-Serve, Cambridge, Mass.
Survival of Cr\(^{51}\) labeled erythrocytes: Two ml. of blood were collected through a heparinized capillary glass tube in 0.4 ml. of 1.5 per cent EDTA in 0.15 N sodium chloride containing \(20 \mu\text{C} \text{Cr}^{51}\). The blood was incubated at room temperature for 30 minutes. Thereafter the erythrocytes were washed once in saline. The Cr\(^{51}\)-labeled erythrocytes were then incubated with 5 volumes of autologous serum at 4 C. for 1 hour, washed once in warm saline and reinjected into the ear vein of the donor rabbits. Blood was obtained at suitable intervals for estimation of radioactivity. Blood volume determinations were done by the isotope dilution method and the percentage of circulating labeled erythrocytes was estimated. The T\(_{1/2}\) Cr\(^{51}\) erythrocyte survival in 10 normal rabbits was 14.3 ± 1.2 days. Radioactive counting was done in a well type iodide scintillation counter connected to a 100 channel spectrophotometer* and the time of counting was set to maintain the counting error within 5 per cent.

Preparation of antirabbit sera: The presence of complement on CA sensitized erythrocytes was detected by a guinea pig antirabbit nongamma serum prepared as follows: 5 ml. of pooled rabbit serum were subjected to starch block electrophoresis. The starch block sections containing the \(\gamma\)G and albumin fractions were removed. The remaining starch block was eluted with buffered saline (pH 7.3), and the protein solution was concentrated by vacuum dialysis. Guinea pigs received four injections of this protein solution suspended in Freund's adjuvant at weekly intervals. The pooled immune guinea pig serum was exhaustively absorbed with rabbit \(\gamma\)G globulin obtained by starch block electrophoresis or column chromatography. This antiserum did not react with \(\gamma\)G as demonstrated by immunoelectrophoresis. A polyvalent goat antirabbit serum was commercially obtained.†

RESULTS

The injection of HKLM serotype 4b produced the highest titers of CA (fig. 1). Low CA titers were present in the sera of rabbits injected with HKLM serotype 4a, whereas the antibodies produced in response to HKLM serotypes 1, 2, and 3 had no CA activity. All serotypes produced high titers of specific anti-HKLM agglutinins.

The \(\gamma\)M antibody response: A typical sequence of CA response following multiple HKLM injections is shown in figure 2. A rise in CA was detected in the majority of the sensitized rabbits within 24 hours after completion of the immunization schedule. It increased exponentially and reached a peak titer 5 or 6 days after the injection of antigen. The antibody titer remained constant for 20–25 days, then fell exponentially with a slope comparable to the ascending one. In some animals the CA persisted for 35–45 days.

A second and third CA response was elicited in all rabbits by repeating the immunization procedure at a time when the CA titer was low. The induction period was identical to that observed after the primary antigenic stimulation and peak CA titers were observed 5 days after injection. The antibody level was again static for 20–25 days and then gradually fell. Thus the pattern of CA formation in response to HKLM was virtually identical with each course of antigen. When antigenic stimulation was discontinued the level of CA remained above the preimmunization titer for at least six weeks. All 45 animals sensitized with HKLM serotype 4b responded in the described pattern. The peak antibody titers are given in figure 3.

† Hyland Laboratories, Los Angeles, Calif.
DAYS
8.000 2,000 512.

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# L. MONOCYTOGENES
SEROTYPE

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4A</th>
<th>4b</th>
</tr>
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<tbody>
<tr>
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<td>0</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>COLD AGGLUTININ TITER</td>
<td>0</td>
<td>50</td>
<td>100</td>
<td>150</td>
<td>200</td>
</tr>
</tbody>
</table>

Fig. 1.—CA titers after immunization of rabbits with 5 different serotypes of HKLM. Note high CA titers in animals injected with HKLM serotype 4b.

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RABBITS IMMUNIZED WITH
TYPE SPECIFIC HKLM

REINJECTION
WITH HKLM-4b

COSTEA, YAKULIS AND HELLER

Fig. 2.—Typical sequence of CA and anti-HKLM antibody production in rabbits following multiple antigenic stimulations.

The γG antibody response: The typical γG antibody response to HKLM is shown in figure 2. After an induction period of approximately 6 days, the antibody titer rose slowly and leveled off. A second injection of HKLM at day 31 led to a rapid rise in antibody titer following an induction period of approximately two days.

Immunochemical characterization of the CA: The antibody reacting with erythrocytes at 4 C. was a γM-immunoglobulin as defined by:
(1) **Starch block electrophoresis**: Sera from immunized rabbits were subjected to starch block electrophoresis. The CA activity was found mainly in the $\gamma_1$ zone while anti-HKLM antibodies had the mobility of $\gamma_2$ globulins (fig. 4).

(2) **Sephadex G-200 fractionation**: Figure 5 shows the various protein components isolated by this method. The CA was confined to the first protein peak and thus had the chromatographic behavior of a macroglobulin. Agglutinating anti-HKLM antibodies were found in both 19S and 7S peaks.
Fig. 5.—CA activity of fractions obtained by Sephadex G-200 chromatography.

Fig. 6.—Immunoelectrophoresis of CA eluted at 37 C. from rabbit erythrocytes (upper well). Eluate absorbed at 40 C. with rabbit erythrocytes (lower well). Center trough: goat antirabbit whole serum.

(3) Immunoelectrophoresis: Cold agglutinins were isolated by thermic elution from sensitized rabbit erythrocytes. The eluate was concentrated by vacuum dialysis and subjected to immunoelectrophoresis. The developing antiserum was a goat antirabbit serum. A single arc of precipitation was observed suggesting a single protein species, probably $\gamma$M (fig. 6). No precipitin arc could be detected when the eluate was absorbed with rabbit erythrocytes at 4 C. prior to immunoelectrophoresis.

(4) Treatment with mercaptans: The cold reacting erythrocyte antibody was completely inactivated by incubation with 0.1M 2-mercaptoethanol or 0.1M d,l-penicillamine regardless of the phase of the immune response during which the serum had been collected.

(5) Analytical ultracentrifugation of a serum sample obtained from a rabbit before immunization and 6 days following stimulation with HKLM
Fig. 7.—Ultracentrifugal analysis of rabbit serum: (a) before and (b) after immunization. Serum immuno-electrophoresis developed with goat antirabbit whole serum before (c) and after (d) immunization. Note the well defined γM arc after immunization.

showed a twofold increase in the 19S peak, (fig. 7a and b); the immuno-electrophoretic pattern likewise reflects the increase in the γM-immunoglobulin after antigenic stimulation (Fig. 7c and d).

*Immunocuological characteristics of the γG antibody:* The second variety
of antibody was directed exclusively against the bacterial antigen. The antibody migrated to the $\gamma_2$ globulin zone on starch block electrophoresis. It was present in the second chromatographic peak and was resistant to 2-mercaptoethanol treatment.

**Serologic Characteristics of the CA**

The erythrocytes of 45 rabbits immunized with HKLM serotype 4b were tested with autologous sera obtained before and after antigenic stimulation. The preimmunization titers of CA ranged from 4 to 32, the latter having been present in only 4 animals. After injection of HKLM, the peak titers ranged from 256 to 4096. All rabbits in this series developed elevated titers of cold autoantibodies after immunization (fig. 3).

A high titer CA rabbit serum was tested with erythrocytes from 40 normal rabbits. The agglutination titers at 4 C. against each of these samples was 1024. Thirty different samples of human erythrocytes obtained from hospitalized patients were also tested with the same antiserum. The cold agglutinin titers varied from 256 to 1024. Only one sample of human erythrocytes failed to react; unfortunately, further erythrocyte antigen studies could not be performed on this patient. There was no correlation between the cold agglutinin titer and the blood group of the tested cells. Erythrocytes obtained from sheep, guinea pigs, dogs, rats, mice and oxen did not react with the rabbit antiserum. It is of interest that guinea pigs challenged intravenously with HKLM produced CA reacting with human or rabbit erythrocytes but not with guinea pig erythrocytes.

**Agglutination of Enzyme-Treated Cells**

The possibility that erythrocytes from animal species which did not react with CA could artificially be made reactive was investigated as follows: trypsinized sheep and guinea pig erythrocytes were incubated with HKLM serotype-4b, washed and reacted with rabbit CA at 4 C. and 37 C. Agglutination was observed at both 4 C. and 37 C. and its intensity was not influenced by thermic variations. These results indicate that reactivity at 4 C. with CA is an intrinsic property of erythrocytes of some animal species. This property cannot be transferred to "nonreactive" cells by passive absorption of HKLM antigens.

**Reaction of CA with Cord Blood, I Negative and Paroxysmal Nocturnal Hemoglobinuria (PNH) Erythrocytes**

Eight different samples of erythrocytes obtained from newborn rabbits and 12 samples of human cord blood erythrocytes were incubated at 4 C. with dilutions of rabbit serum having a titer of 512 against adult autologous rabbit erythrocytes. These titers were uniformly low. Erythrocytes from 1 subject found to be I negative were also agglutinated by this antiserum to low levels (table 1). I negative cells absorbed significantly less antibody at 4 C. from sensitized rabbit sera than I positive erythrocytes.

Erythrocytes from a patient with PNH tested with rabbit CA serum were
EXPERIMENTAL PRODUCTION OF CA IN RABBITS

Table 1.—Reactivity of Various Erythrocytes with Rabbit Cold Agglutinins

<table>
<thead>
<tr>
<th>Test Erythrocytes</th>
<th>Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autologous</td>
<td>512</td>
</tr>
<tr>
<td>Allogeneic</td>
<td>512 (10)</td>
</tr>
<tr>
<td>Adult human</td>
<td>256 (1)—512 (4)</td>
</tr>
<tr>
<td>Rabbit—new born</td>
<td>16 (4)—32 (3)—128 (1)</td>
</tr>
<tr>
<td>Human—cord blood</td>
<td>8 (3)—16 (6)—32 (3)</td>
</tr>
<tr>
<td>I negative—human</td>
<td>64 (1)</td>
</tr>
<tr>
<td>Paroxysmal nocturnal</td>
<td></td>
</tr>
<tr>
<td>hemoglobinuria</td>
<td>2048 (1)</td>
</tr>
</tbody>
</table>

agglutinated to a high titer (table 1). In the presence of complement (fresh human or rabbit serum), these erythrocytes were lysed by the rabbit CA to titers of 32, whereas under similar conditions normal adult erythrocytes were lysed to a titer of 4.

The uptake of $\gamma$M antibody by human and rabbit erythrocytes was investigated by means of an $^{125}$-labeled CA fraction obtained by thermal elution from sensitized erythrocytes. One-tenth ml. samples of washed human adult cord blood, PNH erythrocytes, as well as rabbit cord blood and adult erythrocytes were reacted for 2 hours at 4 C. with increments of labeled CA to which heat inactivated normal rabbit serum was added. The agglutinated erythrocytes were gently washed with six changes of chilled buffered saline (pH 7.3) and their specific activity was determined (fig. 8). The absorption of CA by adult human and rabbit erythrocytes was similar and increased as the antibody concentration in the incubation mixture was increased. Human and rabbit cord blood erythrocytes took up only small amounts of antibody from the medium, which is probably a reflection of the low number of antigenic sites on their membranes. PNH erythrocytes had a higher affinity for the labeled antibody at lower antibody concentration than the other types of cells. At high CA concentrations the uptake of labeled antibody on PNH erythrocytes was slightly greater than that of normal human erythrocytes (fig. 8).

Thermic range: Antibody titrations were performed at 4, 10, 15, 20, 27, 30, and 37 C. There was a progressive fall in titer with increased temperature. In general, at room temperature the titers varied from 4 to 32. There was no macroscopically detectable agglutination above 30 C.

Effect of pH on the CA-erythrocyte reaction: The CA reaction as a function of pH was investigated by means of labeled CA isolated by thermal elution. Incubation media were adjusted with isotonic phosphate buffered saline to pH values ranging from 6.1 to 7.9. One-tenth ml. washed rabbit erythrocytes were incubated in the buffered media for 2 hours at 4 C. During the incubation the pH increased slightly by approximately 0.2 and the value at the end of the incubation period was used in the plot (fig. 9). Test erythrocytes were washed three times in cold saline buffered to the pH of the erythrocyte CA incubation mixture before measurement of radioactivity. The maximal antigen-antibody association occurred at pH values of 6.7 to 6.8, and on either side of these pH values the specific activities of the eryth-
Erythrocytes diminished. At 38 C. the radioactive uptake was minimal and was not influenced by pH changes of the incubation media.

**Chemical inactivation of the CA in vivo:** Two rabbits were given two intravenous injections of HKLM serotype 4b on 2 successive days. The cold agglutinin titer rose to 1024 on day 5 and was maintained at this level (fig. 10). One rabbit received, beginning on day 8, daily intramuscular injections of d, l-penicillamine, 50 mg. per Kg. of body weight, whereas the second rabbit served as control. There was a substantial reduction of the cold agglutinin titer while the drug was administered. After discontinuation of penicillamine the CA titer rose to the pretreatment level within 24 hours (fig. 10). In another experiment penicillin G 100,000 units per Kg. of body weight was given intravenously to a rabbit immunized with HKLM. There was no effect on the CA.

**Absorption studies:** The hyperimmune serum used for these absorption studies contained anti-HKLM antibodies of the γM and γG type. The γM antibodies had both HKLM and CA activity (table 2).

One aliquot of hyperimmune rabbit serum was incubated with HKLM serotype-4b for 2 hours at 22 C. and the supernatant tested for CA activity (table 2). No CA could be detected in the serum after this procedure.

Another aliquot was absorbed with 3 volumes of rabbit erythrocytes at 37 C. There was no reduction in antibody titers against HKLM-serotype 4b or rabbit erythrocytes. A third aliquot of the antiserum was incubated at 4 C. with three volumes of erythrocytes for 2 hours. The anti-HKLM antibody of the γM type was absorbed, as indicated by the fact that the supernatant did not contain any antibody which could be inactivated by 2-mercapto-ethanol. No CA against rabbit or human erythrocytes could be detected in
the serum absorbed in this manner. The results of these experiments indicate that closely related antigenic determinants are shared by human and rabbit erythrocytes and HKLM serotype 4b (table 2).

**Absence of Reactivity of \( \gamma \)G Antibodies with Erythrocytes**

The possible role of the \( \gamma \)G anti-HKLM antibody in erythrocyte sensitization was studied with a chromatographically isolated \( \gamma \)G fraction of hyperimmune rabbit anti-HKLM serum. This fraction had no CA activity, although it agglutinated a HKLM suspension to a titer of 1600. This \( \gamma \)G frac-
Table 2.—Anti-HKLM Antibodies and Cold Agglutinin. Absorption Studies

<table>
<thead>
<tr>
<th>Absorbing Antigen</th>
<th>Anti-HKLM Antibodies</th>
<th>Cold Hemagglutinin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>2-Mercaptoethanol</td>
</tr>
<tr>
<td>None</td>
<td>2560</td>
<td>640</td>
</tr>
<tr>
<td>HKLM 4b</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HKLM 4b</td>
<td>4 C.</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit 37 C.</td>
<td>2560</td>
<td>640</td>
</tr>
<tr>
<td>autologous RBC</td>
<td>640</td>
<td>640</td>
</tr>
<tr>
<td>Rabbit 4 C.</td>
<td>640</td>
<td>640</td>
</tr>
<tr>
<td>autologous RBC</td>
<td>4 C.</td>
<td>640</td>
</tr>
</tbody>
</table>

Absorption was labeled with $^{125}\text{I}$ and incubated at 4 C. and 37 C. with human and rabbit erythrocytes. No erythrocyte agglutination was observed at either temperature and no significant absorption of labeled protein could be detected. After incubation with this fraction in the cold for 1 hour, the red cells were washed and reacted with goat antirabbit $\gamma G$ antiserum. This reaction was negative. An attempt was made to absorb the $\gamma G$ fraction with large volumes of rabbit and human erythrocytes at 4 C. and 37 C. The anti-HKLM activity remained unchanged after absorption. The lack of reactivity of the $\gamma G$ serum fraction with erythrocytes was further documented by incubating equal volumes of “reactive” erythrocytes (rabbit and human) and “non-reactive” erythrocytes (guinea pig and sheep) with the labeled $\gamma G$ antibody solution. The specific activities of the incubated erythrocytes were uniformly low. From these experiments we concluded that the $\gamma G$ serum fraction is devoid of cold or warm antiererythrocyte antibody activity.

In vivo effect of the CA: Because of the finding that rabbits, injected with HKLM serotype 4b, developed a mild normochromic, normocytic anemia (as will be described in a separate paper) the conditions under which CA and complement interact to cause hemolysis, were investigated. Only autologous erythrocytes and serum were used in these experiments in order to avoid cross transfusion incompatibilities.

1. Heat inactivated serum (pH 7.3). One volume of Cr$^{51}$-labeled erythrocytes was incubated at 4 C. for 60 minutes with 5 volumes of heat inactivated CA serum. The agglutinated erythrocytes were dispersed at 37 C. and washed once in phosphate buffered saline at 37 C. By this procedure the CA was removed from the red cell surface. The nongamma antiglobulin reaction with a guinea pig antirabbit serum was negative. The labeled erythrocytes were injected into the donor rabbits and 0.5 ml. blood samples were obtained for estimation of radioactivity at daily intervals. Figure 11 shows the erythrocyte survival patterns observed in these rabbits. In all 4 animals the erythrocyte disappearance from the circulation was within normal limits. Thus, in vitro agglutination of erythrocytes in the absence of complement, did not affect the erythrocyte viability.

2. Fresh serum (pH 7.3). Five volumes of fresh serum were used for the in vitro sensitization of the labeled erythrocytes as described above. These erythrocytes gave a weak anti-nongamma globulin reaction, indicating a small amount of complement on their surface. Within 24 hours after injec-
The Cr51 erythrocyte survival after incubation at 4 C. with inactivated and fresh serum, pH 7.2.

Fig. 11.—Cr51 erythrocyte survival after incubation at 4 C. with inactivated and fresh serum, pH 7.2.

Fig. 12—Heat inactivated serum (pH 6.8). Since it was shown that by lowering the pH of the serum in vitro an increased number of γM molecules will react with erythrocyte antigens, the effect of a slightly acidified medium on the erythrocyte survival was tested. Heat inactivated immune serum was acidified to pH 6.8 by the addition of one tenth volume of 0.2N HCl. One volume of Cr51-labeled erythrocytes was incubated for 1 hour at 4 C. with 5 volumes of the acidified inactivated serum. After incubation, the erythrocytes were washed in warm saline (pH 6.8). The nongamma antiglobulin reaction of these cells was negative. In 2 rabbits there was an initial rapid disappearance of 20 to 40 per cent of the injected erythrocytes and a normal survival of the remaining erythrocytes (fig. 12). In 2 other rabbits the
erythrocyte survival time was within normal limits. Thus, a high CA uptake by the erythrocytes at pH 6.8 in the absence of complement altered the viability of these erythrocytes only slightly.

4. Fresh serum (pH 6.8). Labeled erythrocytes were incubated at 4 C. with fresh CA serum adjusted to pH 6.8. The agglutinated erythrocytes were washed once in buffered saline (pH 6.8). The anti-nongamma globulin reaction was strongly positive. The erythrocyte survival was markedly shortened (2-8.4 days) in 4 injected animals (fig. 12).

The results of these experiments show that erythrocytes which had been agglutinated by CA in the absence of complement have an almost normal survival in the circulation and apparently are not damaged by the temporary in vitro attachment of CA. On the other hand, complement is not eluted from the red cell surface at 37 C. and the shortening of the survival time reflects the irreversible damage inflicted upon these cells by complement.
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DISCUSSION

Immunization of rabbits with heat-killed Listeria monocytogenes (HKLM) serotype 4b provokes the synthesis of two types of antibodies. The first type is a cold agglutinin (CA) that reacts with autologous, allogeneic and xenogeneic (human) erythrocytes at 4°C. It is a γM-immunoglobulin. The synthesis of this antibody does not appear to be associated with a “memory” mechanism: its appearance and decay in the animal is the same regardless of the number of antigenic challenges. A similar antibody response has been described for polysaccharide antigens. The usual antibody response to most antigens, however, is characterized by an initial synthesis of γM antibody followed by the synthesis of γG antibodies. The second class of antibodies formed by rabbits challenged with HKLM is a γG-immunoglobulin which reacts with bacterial antigen, but not with erythrocytes. γG anti-HKLM antibodies have no CA activity.

The mechanism of production of the two classes of immunoglobulins in response to HKLM is not clear. It is possible that both antibodies are produced in response to the same antigen and that the serologic differences between them are due to temperature dependent changes which can be induced only in a macroglobulin. It is also possible that these two antibodies are produced in response to two closely related antigens of HKLM. The cross-absorption studies did not solve these questions completely. They demonstrated that the γG antibody reacted only with the bacterial antigen but not with the erythrocytes. The γM antibody reacted with HKLM to the same extent at 37°C. and 4°C. whereas its reactivity with erythrocytes decreased progressively with increase in temperature. The finding that absorption of immune serum with HKLM at 37°C. removes all CA activity, is important evidence in favor of the possibility that the synthesis of both antibodies is provoked either by a single antigen or two very closely related antigens. If this is the case, then rabbit and human erythrocytes must contain either the same or similar antigenic determinants as HKLM-serotype 4b. The erythrocyte antigen could have two possible sources: either it is passively adsorbed to erythrocytes from ubiquitous L. monocytogenes organisms which could be present in all rabbits and most humans, or it is an intrinsic part of the red cell membrane. The first possibility is unlikely for the following reasons: (1) The naturally occurring CA in rabbits and man do not react with HKLM. (2) Rabbit or human erythrocytes passively coated with HKLM are not more reactive with CA than normal uncoated erythrocytes. (3) This antigen is lacking from erythrocytes of sheep and rats which might also be expected to be naturally infected with L. monocytogenes. (4) Artificial coating of such “nonreactive” erythrocytes does not render them susceptible to the CA. (5) In addition, we have recently obtained experimental evidence that Cr<sup>51</sup>-labeled HKLM was rapidly removed from the blood after its injection and none could be detected on the circulating erythrocytes.

The possibility could be considered that the erythrocytes participate as
Table 3.—Comparison between Rabbit and Human Cold Agglutinins

<table>
<thead>
<tr>
<th>Rabbit Cold Agglutinin</th>
<th>Human Cold Agglutinin</th>
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</thead>
<tbody>
<tr>
<td>Class of protein</td>
<td>γM-Immunoglobulin</td>
</tr>
<tr>
<td>Thermal range</td>
<td>2 C.–25 C.</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>6.6–6.8</td>
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<tr>
<td>Complement</td>
<td>Dependent</td>
</tr>
<tr>
<td>Specificity</td>
<td>Anti-HKLM 4b</td>
</tr>
<tr>
<td></td>
<td>Low reactivity with</td>
</tr>
<tr>
<td></td>
<td>cord blood and I</td>
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<tr>
<td></td>
<td>neg. erythrocytes.</td>
</tr>
<tr>
<td></td>
<td>High reactivity with</td>
</tr>
<tr>
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<td>PNH and trypsinized</td>
</tr>
<tr>
<td></td>
<td>erythrocytes.</td>
</tr>
<tr>
<td>In vivo activity</td>
<td>Hemolytic</td>
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</table>

This was not the case in our system because an excess of bacterial antigen inhibited rather than enhanced the binding of antibody to erythrocytes. Damaged erythrocytes might become antigenic in a similar way as liver cells of rabbits injected with Eimeria stiedae12 or carbon tetrachloride.13 In these instances, stimulation of antibody production was probably secondary to the release into the circulation of antigens from damaged liver cells. Since HKLM, when injected into the circulation, neither coats the erythrocytes nor shortens their survival, this possibility is unlikely. It, therefore, seems reasonable to conclude that rabbit and human erythrocytes and HKLM serotype 4b share an antigenic configuration.

The antigen appears to be present in low concentration on the cord blood erythrocytes of rabbit and man as demonstrated by their low reactivity with rabbit CA. In this respect it is of interest that human cord blood erythrocytes and erythrocytes from exceptionally rare adults which, on the basis of their low reactivity with some human autoimmune CA, have been designated as I negative,14,15 also react with the rabbit CA only to low titers.

One of the striking features of the rabbit CA is its great similarity to the CA found in human disease (table 3). Lewis, Dacie, and Szur have shown that complement and optimal pH are required for the demonstration of the hemolytic effect of CA in man.16 These observations also apply to rabbit CA (fig. 11 and 12).

Since rabbit CA react with autologous erythrocytes the question of autoimmunization should be considered. According to present concepts a self-perpetuating autoimmune state could be triggered by breakdown of tolerance to self-antigens. Since CA could be repeatedly produced by stimulation with HKLM, a xenogeneic antigen, breakdown of tolerance to autologous erythrocyte antigens is unlikely. Rather it would appear that the synthesis of antibody with autoreactivity in response to a structurally related bacterial antigen is operative in this instance.17,18

The question of which cell is responsible for CA production as well as the
hematologic sequels of the presence of circulating CA will be dealt with in a future communication. It should, however, be stated that we have observed in the peripheral blood of animals with high titers of CA mononuclear cells with cytologic characteristics similar to those which André et al. described in the regional lymph nodes of rabbits challenged with tissue antigens.20

In man CA may occur following atypical pneumonia and in the course of infectious mononucleosis or certain lymphoproliferative diseases. In the latter CA may be produced by immunologically competent cells which have lost the capacity of recognition of antigens, or autonomously produce immunoglobulins which accidentally fit erythrocyte receptor sites.21 The self-limited CA response following the infection with the Eaton agent in man could be likened to the cold hemagglutinin produced in rabbits sensitized with HKLM.

**Summary**

The kinetics, serologic and immunochemical characteristics of cold agglutinins (CA) produced in rabbits by the intravenous injection of HKLM serotype 4b have been studied.

The cold agglutinin was a γM-immunoglobulin. It agglutinated autologous, allogeneic and human erythrocytes at 4 C. In the presence of complement the CA was hemolytic in vitro and in vivo. The relevance of these observations to concepts of autoimmunity is discussed.

**SUMMARIO IN INTERLINGUA**

Esseva studiate le cinetica e le characteristicas seroiogic e immunochimic del cryo-agglutininas producute in conilios per le injection intravenose de thermo-occidite Listeria monocytogenes serc typo 4b.

Le cryo-agglutinina esseva un immunoglobulina γM. Illo effectuava le agglutination de autologe, allogene, e human erythrocytos a un temperatura de 4 C. In le presentia de complemento, le cryo-agglutinina esseva hemolytic in vitro e in vivo. Le signification de iste observationes pro conceptos de autoimmunitate es discutite.

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Experimental Production of Cold Agglutinins in Rabbits

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