IMMUNE RESPONSE OF A LIPOSOMAL MODEL MEMBRANE

By James A. Haxby,* Constance B. Kinsky, and Stephen C. Kinsky

DEPARTMENT OF PHARMACOLOGY,
WASHINGTON UNIVERSITY SCHOOL OF MEDICINE, ST. LOUIS, MISSOURI

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Model membranes have, in recent years, become increasingly attractive experimental systems for the investigation of various membrane-associated phenomena. Previous studies with the polyene antibiotics have demonstrated a marked parallelism in the response of natural membranes and model membranes (monolayers, bilayers, and liposomes) to these agents.[1-4] During these investigations, it was observed that the potent polyene antibiotic, filipin, produced pits in erythrocyte membranes and lecithin-cholesterol liposomes.[3] These pits (seen in the electron microscope after negative staining with phosphotungstate) were apparently similar to the pits found in membranes from erythrocytes and bacteria which had undergone immune lysis in the presence of complement.[5,6] To test the possibility that the terminal stages in immune and polyene-induced lysis may be related, we have prepared a liposomal model membrane which responds to antibody and complement. The present paper describes the preparation and some of the properties of this system, and its applicability to the study of complement lytic mechanism.

Materials and Methods.—Preparation, extraction, and fractionation of membranes: Membranes were isolated from sheep or beef erythrocytes by the method of Dodge, Mitchell, and Hanahan.[7] The membranes were washed extensively with cold water by suspension and centrifugation (25,000 × *g* for 20 min) to remove nonlipid materials which contain phosphate. Extraction and fractionation were carried out by a modification of the Bligh-Dyer procedure.[8] To the aqueous suspension of membranes were added 2.5 vol of methanol followed by 1.25 vol of chloroform. After being stirred for 1 hr at room temperature in a closed flask under N_2, insoluble material was removed by centrifugation (30,000 × *g* for 10 min). The clear single-phase supernatant solution was next treated in one of two ways: (*a*) removal of the organic solvents by evaporation and concentration under reduced pressure to yield an opalescent suspension, designated fraction I, or (*b*) further solvent fractionation. The latter was accomplished by the addition of 1.25 volumes of chloroform and 1.25 volumes of water ("volumes" refers to the initial membrane suspension). After centrifugation in a swinging bucket rotor (*ca.* 2,000 × *g* for 20 min), 2 phases, which were separated by a layer of interfacial material, were apparent. The upper methanol-water phase was carefully removed by aspiration and concentrated to yield fraction II*b*. To the tubes containing the interfacial material and lower chloroform phase, approximately 5 volumes of hexane were added. The purpose of this was to reduce the specific gravity of the lower phase to permit sedimentation of the interfacial material and residual aqueous phase. After centrifugation (2,000 × *g* for 20 min), the supernatant solution was taken to dryness under reduced pressure; the residue was redissolved in chloroform to give fraction II*a*.

Preparation of liposomes: These were made essentially by the "micromethod" as described previously.[4,9] An aliquot of one of the above fractions containing 2 μmoles of organic phosphate (or, in the case of the artificial lipid mixtures, 2 μmoles of phospholipid) was added to 10-ml conical flasks. The solvent was removed under reduced pressure followed by evacuation (in a desiccator kept at 0.05 mm Hg) for 1 hr. Marker solution (0.2 ml of 0.3 *M* glucose) was added to the flask and the dried residue was dispersed by agitation with a Vortex mixer. In some experiments, particularly those involving

fraction I, a minute quantity of glass beads (0.2 mm in diameter) was present during agitation to ensure adequate displacement of material from the walls of the flask. The liposome preparations were subsequently dialyzed for 1.5 hr at room temperature against 150 ml of isotonic salt solution (0.075 *M* NaCl–0.075 *M* KCl) to remove most of the untrapped marker.

Assay for glucose release in the presence of AS and C′ (see footnote†): Release of glucose from the liposomes was followed by the spectrophotometric procedure previously used to determine the effect of filipin and temperature on liposomal permeability.[4, 9] These papers may be consulted for details of the method and its validation. In the present investigation a standard assay reagent was prepared which contained: 2.0 mM ATP, 1.0 mM TPN^+, 3.5 mM $MgCl_2$, 145 mM NaCl, 0.15 mM $CaCl_2$, 4.9 mM Veronal (pH 7.4), and approximately 80 μg hexokinase and 41 μg glucose-6-phosphate dehydrogenase per ml. 0.5 ml of the assay reagent was added to each experimental cuvette; control cuvettes contained 0.5 ml of a reagent which was identical to the above except that TPN^+ was omitted. Appropriate quantities of Veronal-saline (145 mM NaCl, 4.9 mM Veronal, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$; final pH, 7.4), liposomes (5 μl were routinely taken for assay), C′, and AS were then added in the order indicated to give a total volume of 1 ml; the cuvettes were incubated at room temperature (*ca.* 23°). The basis for the assay method is illustrated by the experiment described in Figure 1. Addition of liposomes at zero time produced a slight increase in absorbance at 340 mμ which, as shown previously,[4, 9] was due to the small amount of untrapped glucose that had not been removed by dialysis. Similarly, addition of C′ at 2 min caused a further small increase in absorbance due to endogenous glucose remaining in the dialyzed guinea pig serum used as the source of C′ (see below). However, when AS was added at 5 min, a significant increase in absorbance occurred (0.545 optical density units). Under identical con-

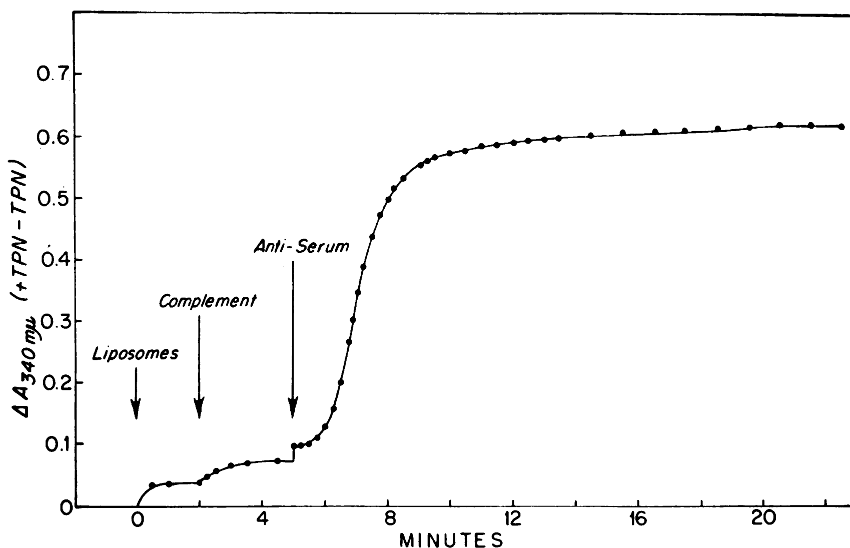

FIG. 1.—Spectrophotometric assay for glucose release from liposomes in presence of AS and C′. At zero time, 5 μl of liposomes (prepared from sheep fraction II*a*) were added to experimental ($+TPN^+$) and control ($-TPN^+$) cuvettes containing 0.5 ml of the appropriate assay reagent and 0.43 ml of Veronal-saline. After 2 min, 53.8 μl of guinea pig serum (containing 7.8 $C'H_{50}$ units) and, after 5 min, 11.1 μl of a 1:10 dilution of AS were added to each of the cuvettes. The curve indicates the difference in absorbance between the experimental and control cuvettes after correction for dilution following each addition. See text for further details.

ditions, the amount of AS used in this particular experiment caused an increase of only 0.032 in the absence of liposomes. Accordingly, after correction for the changes caused by the addition of liposomes, C′, and AS, the difference in absorbance (0.513) between the experimental and control cuvettes served as a measure of the amount of glucose released from the liposomes in the presence of AS and C′. The results below are expressed as the per cent of the total available trapped glucose released within 30 min following AS addition. For this purpose, the total amount of trapped marker present in each liposome preparation was determined by the change in absorbance at 340 mμ after complete lysis of the liposomes with 1% Triton.[4, 9]

Miscellaneous: The cofactors, enzymes, and lipids were obtained from the same commercial suppliers as in previous studies.[4, 9] Hexokinase and glucose-6-phosphate dehydrogenase were extensively dialyzed against distilled water before use in the spectrophotometric assay. Sheep and beef blood, rabbit anti-sheep hemolysin (AS), and fresh frozen guinea pig serum were purchased from the Colorado Serum Co., Denver, Colorado. The level of endogenous glucose in the guinea pig serum was reduced by dialysis at 4° for 5 hr against 200 volumes of Veronal-saline (see above); the solution was changed hourly. Immediately after dialysis, an aliquot was assayed for hemolytic activity ($C'H_{50}$ units) by the method of Mayer[10] and the remainder stored at −80° until used. Normal and immune rabbit sera were decomplemented by heating at 56° for 30 min. Total phosphate and protein were measured by minor modifications of the procedures of Gerlach and Deuticke,[11] and Lowry *et al.*,[12] respectively.

Results.—Release of glucose in the presence of antibody and complement: Liposomes, containing appreciable quantities of trapped glucose, could be readily prepared from sheep fractions I and II*a* (Table 1). Liposomes could not be made with fraction II*b*, which is in accord with the finding that this fraction lacks the main phospholipids, sphingomyelin and phosphatidyl ethanolamine, present in sheep erythrocytes (see below). Incubation of liposomes, prepared from fractions I or II*a* with AS in the presence of C′, under the conditions described in *Materials and Methods*, produced a marked release of glucose (Table 1). Subsequent investigation of this phenomenon revealed many features in common with the phenomenon of immune lysis:

(*a*) Release of marker apparently involves the participation of antibody. Thus, when serum from nonimmunized rabbits is employed instead of the sheep hemolysin, almost no marker is lost from the liposomes (Table 1). Glucose re-

TABLE 1. *Glucose release from different liposome preparations in the presence of AS and C′.*

Fraction used for liposome preparation	Glucose trapped (mμmoles/5 μl liposomes)	Trapped glucose released with immune serum* + C′ (%)	Trapped glucose released with nonimmune serum + C′ (%)
Sheep I	144	73.4	5.2
Sheep II*a*	132	64.2	5.3
Beef I	63	6.9	5.4
Beef II*a*	148	3.7	4.6
SM/Chol/DCP	166	7.3	
as above + 468 μg sheep II*b*	74	52.6	
PC/Chol/DCP	92	8.2	
as above + 468 μg sheep II*b*	53	35.1	

Glucose release was determined in the presence of 1.11 μl AS (*rabbit anti-sheep erythrocyte serum) or serum from nonimmunized animals, and 7.8 $C'H_{50}$ units. In experiments with liposomes prepared from lipid mixtures containing sphingomyelin (SM) or phosphatidyl choline (PC), the molar ratios of phospholipid:cholesterol:dicetyl phosphate (DCP) were 2:1.5:0.22, respectively.

lease is negligible in the absence of AS and saturation is obtained with increasing amounts of AS (Fig. 2).

(*b*) Similarly, release of marker is dependent on the presence of unheated guinea pig serum. Moreover, the response to increasing amounts of C′ is sigmoidal (Fig. 3); this is analogous to the response to C′ concentration when it is titrated in the hemolytic assay.[10] It seems particularly significant that the treatment which is commonly used to inactivate C′ in the hemolytic assay (i.e., heating for 30 min at 56°) also abolishes any release of glucose even in the presence of optimal AS concentrations (Figs. 2 and 3).

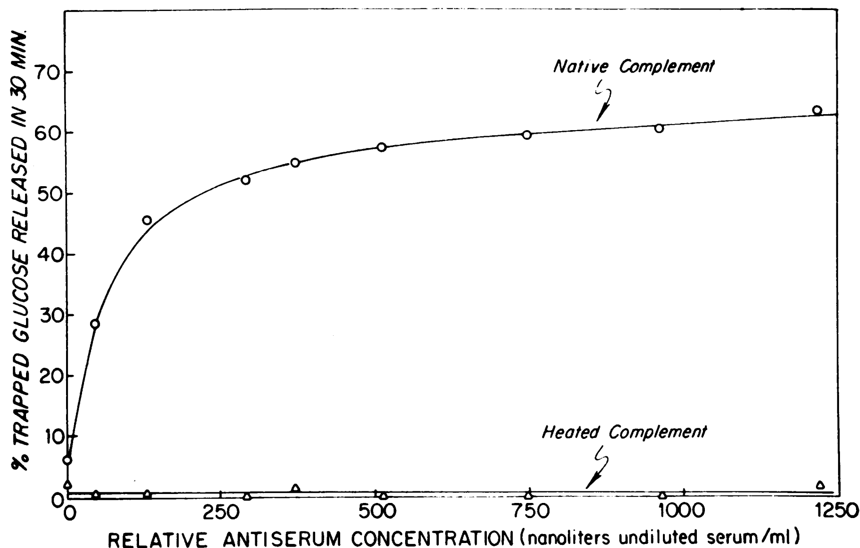

FIG. 2.—Effect of rabbit anti-sheep erythrocyte serum (hemolysin) concentration on release of glucose. Liposomes were prepared from sheep fraction II*a* and assayed in the presence of 7.8 $C'H_{50}$ units and various amounts of AS.

(*c*) The levels of AS and C′ used in the preceding experiments approach, in order of magnitude, the amounts employed in the hemolytic assay.[10] Under these conditions, we have consistently observed a lag in the release of glucose (Fig. 1) which mimics the delay in the onset of hemolysis encountered in the hemolytic assay.[10]

Analysis of fractions: The above experiments were performed with fraction II*a*, but it should be noted that identical results were obtained with liposomes prepared from sheep fraction I. The phosphate and protein composition of these fractions is summarized in Table 2. The considerable loss in dry weight following extraction of the ghosts is due to the insolubility of membrane protein in the chloroform: methanol: water mixture. Preliminary experiments have demonstrated the effectiveness of the washing procedure in removing the inorganic phosphate which constitutes the hypotonic buffer employed in the preparation of the erthrocyte membranes (see *Materials and Methods*). Accordingly, determination of total phosphate serves as a reliable measure of organic phosphate;

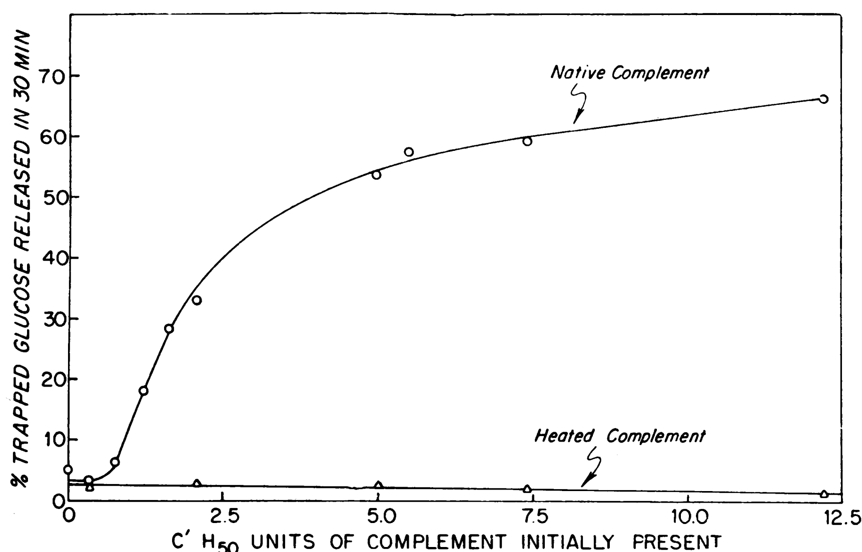

FIG. 3.—Effect of guinea pig serum (complement) concentration on release of glucose. Liposomes were prepared from sheep fraction II*a* and assayed in the presence of 1.11 μl AS and various amounts of C′.

most of this is present in the lipids (i.e., soluble in chloroform, fraction II*a*). Chromatography of fraction II*a* on silica gel plates with chloroform: methanol: water (65:35:4), followed by visualization with iodine vapor and ninhydrin spray, indicated the presence of phosphatidyl ethanolamine, phosphatidyl serine, sphingomyelin, and trace amounts of phosphatidyl choline. The presence of phosphatidyl serine may partially account for the ability to prepare liposomes containing trapped marker without further addition of a charged amphiphile to fractions I and II*a*. In some fractionations, a trace amount of phosphatidyl serine (but not any other phospholipid) could be detected in fraction II*b* also. The main neutral lipid present in fraction II*a* was cholesterol.

Reconstitution of liposomes sensitive to antiserum and complement with an artificial lipid mixture: Previous detailed analyses of sheep erythrocyte membranes by de Gier and van Deenen,[13] and by Maheswaren and Lindorfer,[14] give the following approximate lipid composition (expressed on a molar basis): sphingomyelin,

TABLE 2. *Analyses of fractions obtained from sheep erythrocytes.*

Fraction	Volume (ml)	Total dry weight (mg)	Total phosphate (μmoles)	Total protein (mg)
Membranes	100	548	200	239
I	18.5	129	95	4.6
II*a*	10.0	96	68	0*
II*b*	10.0	15.6	8.0	1.1

See *Materials and Methods* for details. The volume of the initial chloroform: methanol: water extract was 415 ml. 225 ml of the extract were concentrated to give fraction I and 190 ml were fractionated to obtain fractions II*a* and II*b*. The combined recoveries in these fractions were: phosphate, 86%; dry weight, 44%; protein, 2.5%.

* The protein assay used would have detected 10 μg of protein per ml of fraction II*a*

22.2%; phosphatidyl ethanolamine, 17.2%; phosphatidyl serine, 0.6%; phosphatidyl choline, 0.4%; cholesterol, 49.0%. When liposomes were prepared from a mixture containing these lipids in the above molar ratios, glucose was trapped but no significant release occurred in the presence of AS and C′. However, marker was released when small amounts of sheep fraction II*b* were incorporated into the mixture used for preparation of the liposomes. Subsequent experiments demonstrated that the lipid mixture need not be as complex as indicated above. Thus, liposomes made from sphingomyelin (or phosphatidyl choline), cholesterol, and dicetyl phosphate released appreciable amounts of glucose but only when fraction II*b* was also incorporated (Table 1 and Fig. 4). As in the case of lipo-

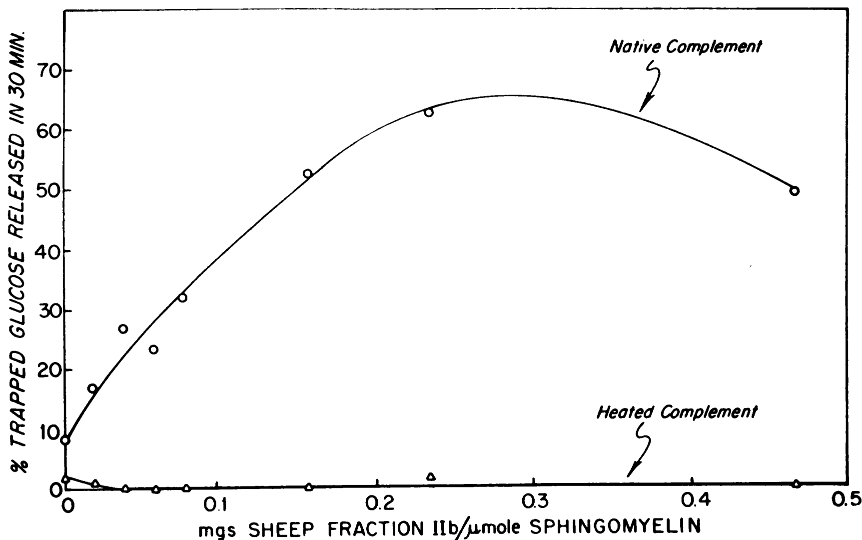

Fig. 4.—Effect of increasing amounts of sheep fraction II*b* on the release of glucose from "sphingomyelin-cholesterol" liposomes. Liposomes were prepared with various amounts of fraction II*b* added to a lipid mixture containing sphingomyelin: cholesterol: dicetyl phosphate in molar ratios of 2:1.5:0.41, respectively. The abscissa indicates the mg of fraction II*b* per μmole of sphingomyelin in the liposome preparation. The liposomes were assayed in the presence of 1.11 μl AS and 7.8 $C'H_{50}$ units.

somes prepared from sheep fraction II*a*, it was also observed that marker was not released from these "artificial" liposomes when the guinea pig serum had been heated to inactivate C′ (Fig. 4).

Nature of the antigen: The preceding experiment indicates that the antigen(s), which accounts for the immune response of the liposomes, is present in fraction II*b*. In the particular experiment illustrated in Figure 4, some inhibition was observed with high amounts of fraction II*b*. This may indicate that not all of the antigen was incorporated into the liposomal structure; the free antigen may possibly have neutralized and fixed a portion of the antibodies and C′, respectively, rendering them inaccessible to the liposomes. It should be emphasized that the identity of the antigenic component(s) must still be established but the available evidence suggests Forssman antigen. It seems unlikely that protein is

the responsible antigen because sheep fraction II*a*, which lacks detectable protein (Table 2), is sensitive to AS and C′ (Table 1). Papirmeister and Mallette have noted that beef red blood cells do not contain Forssman antigen.[15] Accordingly, if Forssman antigen is involved, it would be expected that liposomes prepared from fractions I or II*a* derived from beef erythrocyte membranes should not release significant amounts of marker in the presence of AS and C′. This prediction was confirmed (Table 1).

Discussion and Summary.—In this paper, we have described the preparation of liposomes in which certain features of immune lysis apparently can be reproduced. This is not the first example of an effect of antigen-antibody interaction on a model membrane. In 1966, del Castillo and co-workers observed that complex formation between certain antigens (e.g. insulin) and their appropriate antibodies produced a marked decrease in the electrical resistance of thin lipid bilayers.[16, 17] This effect was, however, obtained in the absence of C′ and may not reflect a genuine "immune response" of the model membrane because analogous resistance changes resulted when, for example, certain enzymes (such as lactic dehydrogenase) combined with their substrates. More recently, Barfort *et al.*[18] described the preparation of a bimolecular lipid film that required the addition of unheated guinea pig serum in order to achieve a resistance decrease in the presence of antibody and antigen. Their observations, as well as those reported in this paper, suggest that model membranes may be exceedingly useful for studying complement mechanism.

As an experimental tool, however, thin lipid (secondary black) films still leave much to be desired. In contrast to the liposomal model membranes introduced by Bangham and co-workers,[19, 20] stable and responsive bilayer films cannot always be obtained; they are not convenient objects for examination by negative-staining electron microscopy, X-ray, or NMR spectroscopy, and the lipid composition of the films may not be identical to the composition of the mixture used to prepare the membranes. In this connection, the advantages of being able to prepare liposomes with pure lipids should be noted. For example, it has often been suggested that the terminal stages in immune lysis may involve degradation of one or more of the membrane phospholipids and this alternative can be readily tested with the liposomal model membrane. Indeed, the possibility that lysolecithin (originating from the membrane) may be an important intermediate would seem to be excluded by the experiments which have shown that "sphingomyelin-cholesterol" liposomes release appreciable amounts of marker.

However, there are some obvious experimental deficiencies which must first be eliminated. For example, we do not yet know whether all the components of C′, required for immune lysis, are also necessary to induce glucose release from liposomes in the presence of AS. In fact, the possibility that heat treatment may have inactivated some unknown factor (not related to C′) in the guinea pig serum, must still be considered. The availability of purified C′ components, as well as procedures which specifically inactivate some of these components, should answer this question. Similarly, the assumption that Forssman antigen is responsible for the sensitivity of the liposomes to sheep hemolysin can be tested using the purified antigen described by Rapp and Borsos.[21]

Finally, it should be noted that the procedures described in this paper may also be useful in preparing liposomes which contain other amphipathic antigens. This approach is now being tested with a variety of "smooth" and "rough" lipopolysaccharides from bacteria. In this regard, the recent observations of Mergenhagen *et al.*[22] appear extremely significant. They have shown that fixation of certain C′ components by lipopolysaccharide produces lesions which resemble the pits found in erythrocyte and bacterial membranes after immune lysis. We have found similar pits in liposomes that are rapidly releasing glucose in the presence of AS and C′ (manuscript in preparation). These observations, together with the experiments described above, further support the feasibility of employing liposomal model membranes to study C′ mechanism.

Note added in proof: Since submission of this manuscript, additional evidence, which indicates the involvement of complement, has been obtained. Significantly less glucose was released from liposomes in the presence of guinea pig serum which had been partially depleted of components C′1, C′4, and C′3, by reaction with an immune precipitate. It has also been found that liposomes fix C′ in the presence of AS and are able to neutralize anti-Forssman antibodies.

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† The following abbreviations are used: AS (rabbit anti-sheep erythrocyte serum or sheep hemolysin); C′ (complement).

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