OXYGEN-STABLE HEMOLYSINS OF GROUP A STREPTOCOCCI

IV. Studies on the Mechanism of Lysis by Cell-Bound Hemolysin of Red Blood Cells and Ehrlich Ascites Tumor Cells*

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In the previous paper (1) data were presented on the relationship of the cell-bound hemolysin (CBH) of a Group A streptococci to the oxygen-stable hemolysin produced by these organisms in the presence of RNA. It was shown that a number of agents and conditions affected similarly the formation and activity of the cell-bound and RNA-induced hemolysins. The fact that (in the absence of an effective inducer of the oxygen-stable hemolysins, such as RNA) no traces of soluble hemolysin can be detected in the suspending medium of washed streptococci even with high CBH activity suggested that in the absence of such an inducer a hemolytic moiety can be transferred directly from the streptococci to the red cells, as has, in fact, been indicated in a preliminary report (1). The hemolysin thus transferred would act on some cell membrane component to cause the permeability changes which result in the lysis of the cells. However, the actual mechanism of lysis of red blood cells (RBC) and other mammalian cells by CBH (2) and the oxygen-stable streptococcal hemolysins is not understood.

In the present paper experiments on the mechanism of lysis of red blood cells by washed streptococci possessing CBH activity are reported, and a proposed relationship between the CBH and the group of streptococcal oxygen–stable hemolysins is presented.

Materials and Methods

The streptococcal strain, culture media, and the preparation and titration of RNA and the cell-bound hemolysin were described in a preceding paper (1). Group O human red blood cells were used throughout.

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Ehrlich ascites tumor cells were harvested from the peritoneal washings of Swiss mice injected with the Landchutz strain of tumor cells. Cell obtained 7 days after the injection were washed with buffered saline and resuspended at 5×10^7 cells per ml.

The preparation of cellulose columns will be described below.

RESULTS

A. The Possible Role of Streptococcal and Red Blood Cell Supernates in the Induction of Hemolysis by Streptococci Possessing CBH Activity.—As has been previously shown, the production of oxygen-stable hemolysins can be induced by quite a wide variety of molecular species (RNA, serum albumin, and α-lipoprotein, tween, triton, lecithovitellin, and trypan blue) (1). Also, the lysis of RBC by washed streptococci (in the absence of these inducers or carriers) probably does not involve reagents other than maltose, Mg^{++}, and SH-compounds, but rather only the interaction between the RBC and streptococci, no free hemolysin being observable in the supernates of the bacterial suspensions. Because of these facts it was considered important to examine the possibility that some component spontaneously released from the streptococcal cell or from the RBC, or released in the course of interaction of the two cell types, might have some function in the induction of hemolytic activity.

The following experiments were carried out on such materials. In none of these was any evidence obtained of induction of hemolytic activity by the streptococci, or even of increase in the degree of hemolysis in an effective system.

1. Incubation of RBC with supernates obtained from thick streptococcal suspensions possessing CBH activity. Supernates from such reaction mixtures were incubated with streptococci in the presence of the activation mixture: maltose, Mg^{++}, and cysteine (AM) and the supernates of these reaction mixtures were analyzed for hemolytic activity.

2. Incubation of streptococci in AM with washings of RBC.

3. Incubation of streptococci with hemolysates of RBC obtained by the use of distilled water or CBH.

4. Incubation of RBC with urea extracts of washed streptococci. Such extracts have been shown by Krumwiede (4) to contain lipoproteinase activity, and it was considered that the enzyme might release a lipid component from red cell lipoproteins, or from lipoproteins adsorbed to the red cells, which could serve as a carrier of the hemolysin, in a manner similar to that demonstrated by serum lipoproteins or by lipids bound to serum albumin (3). A sample of this enzyme, partially purified by Rowen (5), was made available through the courtesy of Dr. Rowen.

5. Incubation of RBC with streptococci of strain C203U. This strain, a mutant of C203S, has been shown not to produce either RNA hemolysin or CBH (6) in contrast to the parent strain. The supernates from incubation mixtures of strain C203U and streptococci were incubated with streptococci of strain S84 and RBC in the presence of the AM. No increase in degree of hemolysis was observed in comparison with the control mixture of S84 streptococci, RBC, and AM.

B. Experiments on the Separation of RBC from Streptococci after Incubation.—Data obtained in earlier studies suggested that the lysis of RBC by CBH in-

volves the transfer of some hemolytic group from streptococci to RBC, but it was not possible to demonstrate experimentally the transfer of such material. Microscopic examination of streptococcal-RBC mixtures did not reveal aggregation between the two kinds of cells, although the red cells were observed through the stages of swelling, sphere formation, and finally ghost formation (Fig. 1 *a* to 1 *c*). In the case of Ehrlich ascites tumor cells incubated with streptococci, a few coccal chains could occasionally be found in juxtaposition to the cell membrane, but it was not possible to determine whether these streptococci were responsible for the cellular damage seen or whether adsorption of streptococci to the cells occurred after they were already damaged by the hemolysin.

The following experiments were carried out on the degree of physical association between streptococci and RBC necessary for eliciting hemolysis, and on the separation of RBC from active streptococci after their interaction.

1. Experiments with Millipore filters: A double glass chamber was prepared, the two parts being separated from each other by a Millipore filter of pore size of 0.22 μ. One part was filled with 3 ml of a streptococcal suspension in AM possessing 2000 hemolytic units (H.U.) per ml, and the other was filled with 3 ml of RBC (1 per cent suspension). In another experiment, one part of a similar double chamber was filled with a solution of RNA hemolysin (10 H.U. per ml) while the other part of one chamber contained the RBC suspension. Both double chambers were placed in a water bath at 37°C. It was found that the red blood cells in the double chamber containing RNA hemolysin were completely lysed after 30 minutes of incubation. The RBC in the double chamber containing CBH were not lysed after 6 hours of incubation. At the end of the experiment, aliquots of streptococci removed from the chambers still possessed considerable hemolytic activity. Thus, separation of streptococci from RBC by a membrane which allows free diffusion of RNA hemolysin prevented lysis of the red cells by streptococcal cells.

2. Experiments with agar tubes: In these experiments 2 ml of isotonic saline solution containing 1 per cent of Nobel agar (Difco Laboratories, Inc., Detroit) and 0.5 per cent of washed RBC were poured at 48°C into a serologic tube and allowed to solidify, after which a layer of agar containing streptococci with 1500 H.U. per ml was poured over the agar-RBC. After the second layer solidified the tubes were incubated at 37°C (tube A). In another tube, B, the two layers containing RBC and streptococci respectively were separated from each other by a 0.5 mm layer of plain agar. After 2 to 4 hours of incubation a narrow zone of hemolysis appeared in tube A, at the interface of the two layers, whereas no visible hemolysis occurred in tube B. Again the hemolysis of streptococci by RBC, in the absence of an inducer, or carrier, of oxygen-stable hemolysin was prevented by the interposition of a layer through which macromolecules could diffuse freely, suggesting again a direct transfer of a hemolytic substance from streptococci to RBC during their incubation.

3. Separation of streptococci from red blood cells after incubation by means of cellulose columns: In the following experiments it was in fact found possible to indicate the transfer of hemolytic material from streptococci to RBC during the incubation of the two, by the separation of the two cell types after a part of the required period of incubation. Carboxymethyl (CM) cellulose powder type 40 (Schleicher and Schüll, Keene, New Hampshire) or plain cellulose (Whatman) was suspended in water and the suspension was poured into a glass cylinder containing distilled water. The heavy particles were discarded, and the medium and small particles were collected and washed several times with saline buffered at pH 7.2 with 0.05 M phosphate. The slurry of cellulose was then poured into glass columns of 1.0 x 10.0 cm over a plug of glass wool

and packed under 5 p.s.i. of air pressure to make a 3-cm-long column of cellulose. Following several washes with buffer, the columns were kept at 4°C and used on the same day.

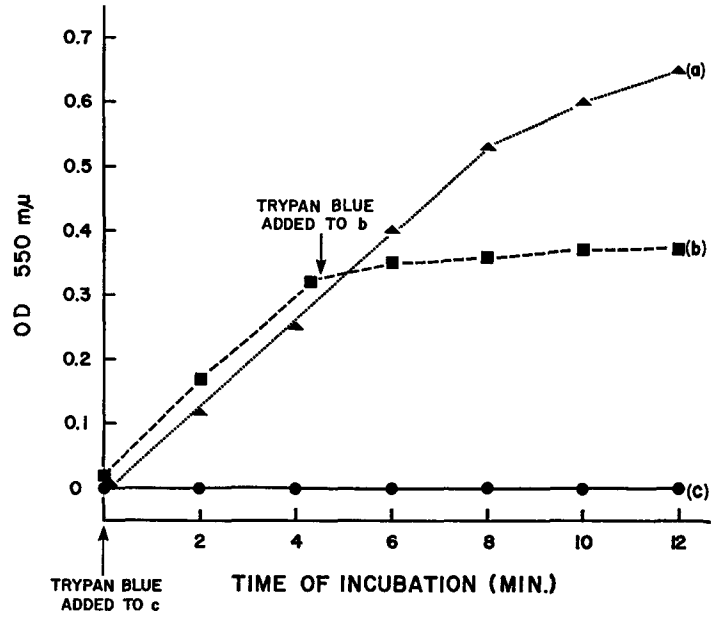
Washed streptococci of strain S84, harvested from the stationary phase of growth, were incubated with maltose, Mg^{++}, and cysteine. Such streptococci had been found to have considerable CBH activity (1, 6). Of such a streptococcal suspension, 0.1 ml was mixed with 0.9 ml of a 10 per cent suspension of human RBC previously washed with saline. The mixture was immediately chilled to 1°C in an ice bath, applied to the cellulose column, and allowed to penetrate into the column, which was then filled with ice cold buffer. Pressure was then applied to the column, and successive portions of the effluent were examined for the presence of streptococci by phase microscopic examination. Such effluents contained either no streptococci or, occasionally a few cocci in an occasional field. (That the trapping of the streptococci is probably mechanical, and is perhaps due to long chain formation of the streptococci was indicated by the observation that streptococci growing in short chains were found to be trapped to a lesser extent, and that no streptococci were eluted from such columns by a 1 M solution of NaCl.) On the other hand, the effluent contained over 95 per cent of the RBC applied to the column, as determined by lysis of the cells in portions of the effluent with saponin. The RBC appeared in little more than the column volume of effluent. When suspensions of the RBC thus separated from the steptococci were incubated at 37°C, lysis of the majority of red blood cells occurred within 30 to 60 minutes The time of lysis of the red cells was inversely related to the time of incubation of streptococcus-RBC mixtures prior to their application on the column.

In order to examine the possibility that lysis of RBC following the separation on the column was due to the few streptococci that were sometimes present, papain at 50 μg/ml, or trypan blue at 0.1 mg/ml, were added to some samples of effluents containing RBC which had been separated from streptococci as described above. (It has been shown elsewhere (1, 6) that papain and trypan blue inactivate CBH when incubated with streptococci prior to the addition of RBC, but do not inactivate the hemolysin after adsorption to RBC.) It was found that the hemolysis of the RBC in these column effluents was not affected by the presence of papain, whereas in the control preparations the inactivation of CBH by papain was again demonstrated. Thus the lysis of the separated RBC could not have been due to contamination by the few streptococci observed in occasional preparations, or by undetected streptococci in the effluent.

4. Kinetic study of the lysis of RBC by CBH: The method for the separation of RBC from streptococci described above made it possible to examine the effect of variations in interval of incubation, prior to the separation, on the amount of hemolysin transferred, as indicated by the time required for lysis of the RBC after separation from the streptococci. In order to study this in more detail, the transfer reaction was reduced in rate by carrying the incubation out at 25°C, since at this temperature lysis by uninterrupted incubation with CBH requires 10 to 15 minutes, in comparison with 2 to 5 minutes at 37°C.

Streptococci were incubated for 5 minutes at 37°C with AM and 25 μg/ml of cholesterol (to inhibit any streptolysin 0 that might be present). Following activation of CBH, a 0.1 ml portion was titrated to determine the potency of the CBH, while another portion was centrifuged and the supernate assayed for the presence of hemolysin. If no trace of hemolytic activity was detected in supernates, the CBH was considered suitable for these experiments. First, preliminary experiments were done to determine the maximum period of incubation feasible at this temperature before separation. To a series of chilled tubes, each containing 0.1 ml of activated streptococci, was added 0.5 ml of a chilled 10 per cent suspension of RBC. The final volume was brought to 1 ml with cold buffer, and after various periods of incubation at 25°C, individual tubes were removed, immediately chilled to 4°C, centrifuged, and the supernate checked for hemolysis. In this experiment hemolysis appeared after 12 minutes. A set of tubes was then set up for the column separations. At zero time and at 2-minute intervals one

tube was removed from the bath, chilled at 4°C and put on a CM cellulose column previously washed with ice cold buffer. This was continued until 12 minutes, the period required in this experiment for the first evidence of hemolysis by CBH in uninterrupted incubation, as indicated above. The RBC separated from steptococci were collected in 4 ml of buffer and immediately transferred to 37°C. Following incubation for 60 minutes, the tubes were centrifuged and the amount of hemoglobin released was determined with a Unicam spectrophotometer at 550 mμ. In other experiments, 100 μg/ml of trypan blue were added to CBH-RBC mixtures, in order to prevent the activity of CBH or to terminate it after incubation for 6, 8, and 10 minutes.

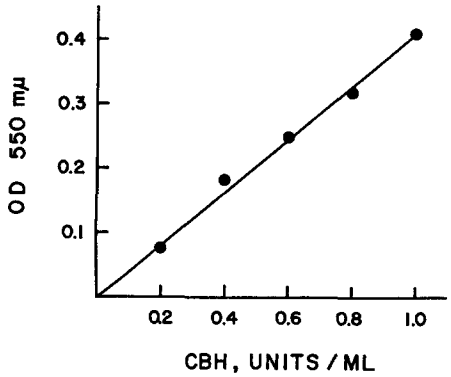


TEXT-FIG. 1. The lysis of RBC separated from CBH-producing streptococci after various periods of incubation. Graphs *b* and *c* show the effects of interrupting and preventing the transfer of CBH, respectively, by the use of a known inhibitor, trypan blue. Graph *a*: no interruption of the incubation.

The data in a typical experiment, presented in Text-fig. 1, show that there was a progressive increase in the degree of lysis of RBC with the increase of time of incubation prior to the separation, indicating a transfer of increasing amounts of hemolysin from streptococci to RBC (*a*). Such a transfer of hemolysin could be terminated (*b*) by the addition of trypan blue, which is known to inhibit the CBH activity of streptococci but not hemolytic activity that has already been transferred to RBC. Finally, trypan blue was found to prevent the transfer of hemolysin completely, if added at zero time (*c*). Similar data were obtained when tetracyclines were used as the inhibitor of CBH activity.

In order to evaluate the data on lysis of RBC after their separation from streptococci in terms of amounts of hemolysin transferred from streptoccci to RBC during the incubation, a series of tubes were set up with RBC in the amounts used in the kinetic experiment and with streptococcal suspensions having known amounts of CBH. After incubation for 1 hour at 37°C the tubes were centrifuged and the OD of the supernate determined at 550 mμ, as in the case of the RBC separated from the streptococci by the cellulose column.

Text-fig. 2 shows that there is a linear relationship between the amount of CBH applied to the RBC and the resulting relative concentration of released hemoglobin. Since the graphs of Text-fig. 1 also showed a linear relationship between the OD of the supernate at 550 mμ and time of incubation of strep-



TEXT-FIG. 2. The relation of the degree of hemolysis (amount of hemoglobin released from lysed RBC) to the relative concentration of CBH-active streptococci with which the RBC were incubated.

tococci with RBC, up to 8 minutes in the uninterrupted incubation (graph *a*) and, in graph *c* up to the interruption of the process by the addition of the inhibitor, it would appear that for the greater part of the process there is a constant rate of transfer of hemolysin from streptococci to RBC.

C. Cytologic Changes Induced in RBC and Ehrlich Ascites Tumor Cells by CBH.—As shown previously, streptococci possessing CBH activity are capable of injuring a variety of mammalian cells *in vitro* (7, 8). Since the lysis of red blood cells by washed streptococci was induced in the apparent absence of extracellular hemolysin, experiments were designed in an attempt to determine whether microscopic evidence would be obtained of physical contact between the streptococci and RBC or ascites tumor cells prior to the lysis, and to make microscopic observations of the changes in the cells in the course of such lysis. Washed human red blood cells were incubated for 1 minute at 1°C with chilled washed streptococci in AM. A drop of the reaction mixture was immediately put on a microscopic slide and a single microscopic field was photographed with an Olympus phase microscope at a magnification of 1000, at 20°C. Pictures were

taken at zero time and at 10-minute intervals thereafter. Fig. 1 *a*, at zero time, shows the suspension of red cells, some of which are somewhat crenated; only few streptococcal chains are visible. Fig. 1 *b*, at 20 minutes, shows the rounding of the cells; some of the cells appear as spheres, and few ghosts can be seen in the preparation. Fig. 1 *c*, taken after 50 minutes, shows many ghosts and cells in different stages of hemolysis. No direct contact between the RBC and streptococci was seen at any time. The effect of CBH on Ehrlich ascites tumor cells is shown in the other three photographs; Figs 1 *d*, 1 *e*, and 1 *f*, at zero time, 30 minutes, and 60 minutes, respectively. (In this case the photographs are not of the same microscopic field.) It can be seen, in Figs. 1 *e* and 1 *f*, that there is progressive swelling of the cells, with large, pseudopod-like structures and the appearance of highly refractile granules both in the nucleus and the cytoplasm. Few streptococcal chains can be seen adhering to the swollen cells.

DISCUSSION

The experiments reported in this paper were performed in order to obtain some data relative to the mechanism of lysis of red blood cells by washed streptococci possessing cell-bound hemolytic activity, and to relate these findings to the general problem of the oxygen-stable hemolysins of streptococci.

The lysis of red cells brought about by direct contact with streptococci under appropriate conditions was first described by Weld (9) and by Smith (10). Neither in their studies nor in more recent ones (1) was it possible to demonstrate any trace of hemolysin in the suspending medium, even of streptococci with considerable CBH activity. The data presented above, which indicate that neither washings or hemolysates of RBC, nor urea extracts of the streptococci studied, contain any material capable of inducing the production of hemolysin by streptococci give further indication that the lysis of RBC by streptococci does not involve an intermediary soluble hemolysin in detectable concentrations. As to the conditions under which streptococci cause hemolysis of red cells, it is apparent that close proximity of CBH-bearing streptococci with RBC is necessary for lysis. This conclusion is based on experiments showing that the separation between streptococci and red cells by means of a Millipore filter or a thin agar layer, which allows a free diffusion of RNA hemolysin, is sufficient to prevent lysis.

Direct evidence for the transfer of hemolytic material from streptococci to red cells was obtained in the experiment showing that when streptococci possessing CBH activity are incubated with RBC for periods of time not sufficient to cause lysis, and then separated from each other by cellulose columns, sufficient hemolytic material is transferred to RBC to cause subsequent lysis in the absence of streptococci.

These observations suggest that a hemolytic moiety is transferred from streptococci to RBC, presumably of so high an affinity for its sites on the surface of either of these cells as to have a maximum concentration in free solution which

is below detectable levels, even in incubation mixtures in which hemolysis occurs.

This hypothesis is consistent with observations recently reported (11) on on oxygen-stable streptococcal hemolysins, and is complementary to a hypothesis, presented in that report, on the relations among the oxygen-stable hemolysins. In that study it was found that a number of properties of the respective hemolysins were identical with those of the inducing molecular species. (An incidental point of further similarity between CBH and the oxygen-stable hemolysins is that no hemolysin free of the inducer could be found in preparations of oxygen-stable hemolysins. For example, in preparations of RNA-induced hemolysin no hemolytic activity could be found, on chromatographic analyses, except in the fraction containing the RNA.) It was found, further, that in certain directions the hemolytic function could be transferred from one inducer, or carrier, to a fresh inducing agent, so that if a mixture of RNA hemolysin and fresh tween was resolved by chromatography, hemolytic activity could now be found in the tween-containing fraction. Finally, in the case of a number of such derived hemolysins it was possible to show that these were not inactivated by agents which could have inactivated the original hemolysin, presumably by hydrolysis or deformation of the original inducing agent. It was suggested that the group of oxygen-stable streptococcal hemolysins constitute a group of molecular species in which a given hemolytic moiety can be bound to any of a group of carrier molecules, these being recognized by their ability to induce the formation of hemolysis from streptococcal cells.

Two kinds of recent evidence give further support to this picture of the interrelation between the postulated carrier molecule and the prosthetic, hemolytic group. First, in experiments reported elsewhere in these studies it was found, after incubation of RNA hemolysin with tween and the subsequent resolution of the mixtures by DEAE cellulose chromatography, that the fraction containing the tween, which had 70 per cent of the hemolytic activity after the incubation, was not contaminated with RNA, within the limits of detection of the orcinol method (11). Second, in experiments reported recently by Egami and Koyama (12), RNA hemolysin labeled with C^{14} amino acids was incubated with tween 40 and the mixture was resolved on DEAE cellulose. Both the C^{14} and the hemolytic activity were divided between the fraction containing RNA and that containing the tween. Again, in these studies, the newly hemolytic tween-containing fraction contained no detectable RNA.

On the basis of the directions in which the hemolytic activity of streptolysin S could be transferred among the inducing species, and of the partition of hemolytic activity between equimolar solutions of inducers, or carriers, the following order of increasing affinity for the hemolytic moiety was indicated, among the four carriers studied: albumin, RNA, tween 40, triton X-205. In view of the similarities between CBH and oxygen-stable hemolysin shown in the previous

paper (1), the absence of free hemolysin in both of these systems, and the evidence for transfer of hemolytic material from streptococcal cell to RBC presented above, it would appear justified to add, to this list of relative affinities for the hemolytic moiety, the binding site on the streptococcal cell wall (at the low-affinity end of the list, since any of the recognized inducers must have a higher affinity for the hemolytic moiety than the streptococcal cell component), and the receptor site on the RBC at the high affinity end (since the hemolysin induced by any of the inducing agents can cause lysis of the RBC).

SUMMARY

In studies of the mechanism of lysis of red blood cells by washed streptococci with hemolytic activity (cell-bound hemolysin, CBH) no components released spontaneously by RBC or streptococci, or by interaction between these cells, could be found to induce the formation of soluble hemolysin by the streptococci. It was also found that separation of RBC from streptococci even by Millipore filter or a very thin layer of agar could prevent their hemolysis.

By means of cellulose columns it was possible to separate RBC from streptococci after a short incubation. RBC thus separated from streptococci with which they had been incubated underwent hemolysis on subsequent incubation at 37°C. By varying the period of incubation prior to separation it was possible to demonstrate the transfer of increasing amounts of hemolysin from streptococci to RBC with increasing periods of incubation. A considerable part of this appeared to be at a constant rate.

A theory is presented on the relationship between the streptococcal cell-bound hemolysin and the group of oxygen-stable streptococcal hemolysins, in terms of a transferable hemolytic moiety and binding sites for this moiety on the streptococcal cell, on various molecular species which can act as inducers of the oxygen-stable hemolysins, and on the RBC, with the affinity of the respective binding sites for the hemolytic moiety increasing in that order.

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EXPLANATION OF PLATES

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FIG. 1 *a* to 1 *f*. Photomicrographs of a field of RBC immediately after the beginning of incubation with active streptococci (Fig. 1 *a*) and after 20 and 50 minutes of incubation (Fig. 1 *b*, and Fig. 1 *c*), and of Ehrlich ascites tumor cells at the beginning of incubation with streptococci (Fig. 1 *d*) and after 30 and 60 minutes of incubation (Fig. 1 *e* and Fig. 1 *f*). × 1000.

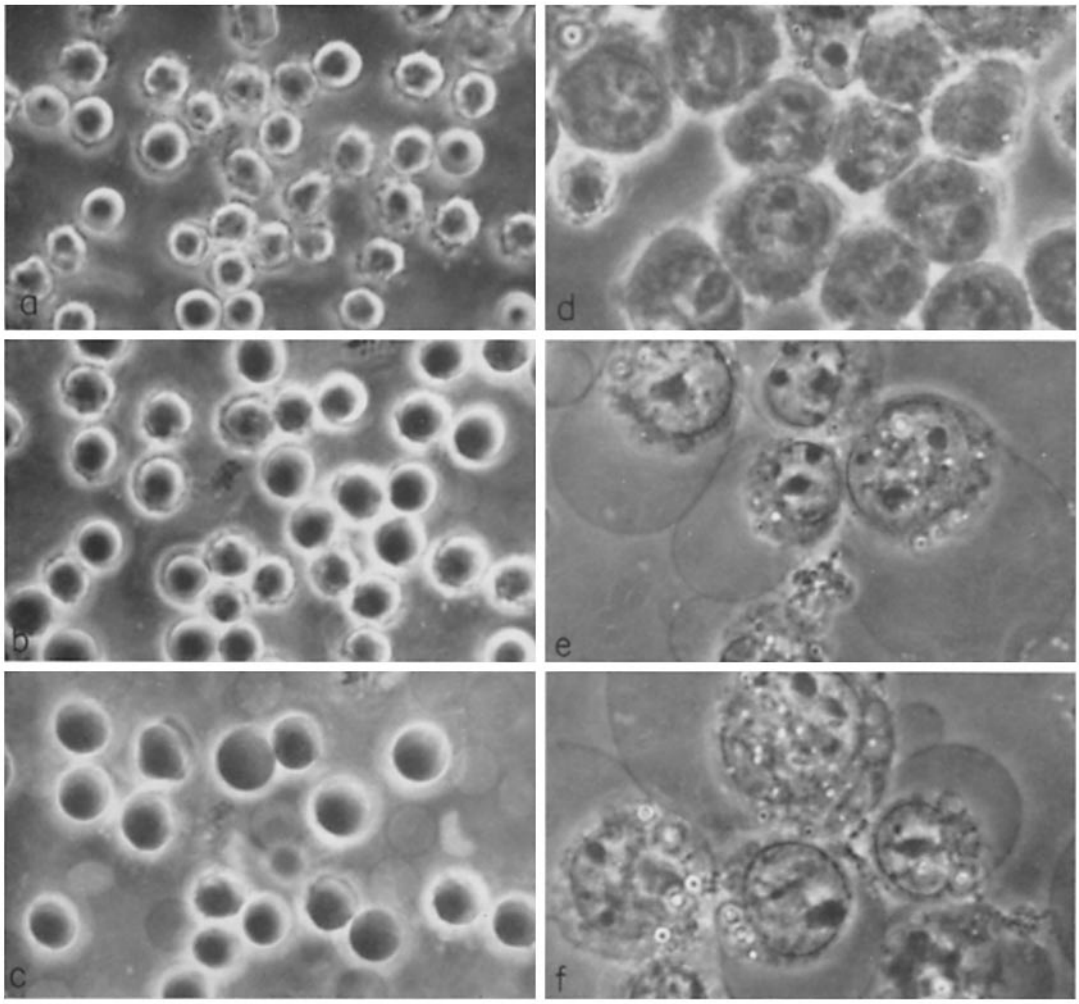

FIG. 1

(Ginsburg and Harris: Hemolysins of Group A streptococci. IV)