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Interaction of Autologous Complement with Red Cells in the Absence of Antibody.*† (29658)

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Significant advances have recently been achieved in relation to chemical characterization and elucidation of the mode of action of serum complement components and their biological significance. One of the important aspects in the study of serum complement is that of the molecular interaction between complement and cell membranes. The study of complement-membrane interaction is usually approached by using the classical hemolytic system consisting of sheep cells coated with non-agglutinating amounts of rabbit antibody, and guinea pig or human serum as a source of complement. In this system the analysis of complement-membrane interaction is complicated by the presence of antibody. This difficulty may be overcome by use of non-specific sensitizers which are capable of mediating the complement reaction without attaching themselves to the membrane.

Among the various substances that have been described as non-specific sensitizers, polyethylene glycol (PG) has been studied most thoroughly(1). McVickar(2) found that this agent is capable of enhancing the hemolytic effect of guinea pig serum on sensitized sheep erythrocytes. Subsequently, Cowan(1) demonstrated that PG can actually substitute for specific antibody in the sheep cell-guinea pig complement system, and that it can thus function as a non-specific sensitizer. He concluded that PG exerts this effect by combining loosely with the red cell membrane and by thus linking complement to its surface.

In the present paper, data are presented which favor an alternative mechanism to explain the effect of PG. To preclude the participation of antibody, all experiments were carried out using an autologous system consisting of human red cells and serum. Analysis of this model has furnished evidence for the occurrence of a direct link between the complement components β_{1E} and β_{1C}-globulin on the one hand and the red cell membrane on the other. In addition, evidence was obtained indicating that membrane-bound neuraminic acid counteracts the attachment of complement components in the absence of antibody. Removal of neuraminic acid from the red cell surface facilitates complement fixation in the PG system.

Materials and methods. Erythrocytes and serum were obtained from healthy white donors. Blood was collected in ACD solution, the plasma and buffy coat were removed and the red cells were washed 3 times with 20 volumes of Ca^{++} and Mg^{++} containing isotonic veronal buffer, pH 7.4. The packed red cells were suspended in the same buffer in a concentration of 2.5%. To obtain serum, a second aliquot of blood was withdrawn from each donor.

Enzymatic treatment of erythrocytes. Washed red cells were suspended in veronal buffer containing 1 mg/ml of either trypsin, chymotrypsin, β-glucuronidase or pancreatic lipase (Worthington Biochemical Corp., Free-

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hold, N. J.), or 20 units/ml of neuraminidase (Behringwerke Ag., Marburg/Lahn, West Germany) at a cell concentration of 5%. They were incubated at 37° for 30 minutes, then washed 3 times and resuspended in veronal buffer. Between the first and the second washing, the trypsin-treated cells were incubated for 10 minutes at room temperature with veronal buffer containing 0.3 mg/ml of soybean trypsin inhibitor (Mann Research Laboratories, New York), at a cell concentration of 5%.

Polyethylene glycol. This synthetic polymer, with the trade name of Carbowax-4000 was purchased from Amend Drug and Chemical Co., New York. A 15% stock solution was prepared in veronal buffer and kept in the cold.

Antisera. Rabbit antisera against highly purified human β_{1E} and β_{1C}-globulin were prepared as previously described(3,4). Antisera against human immunoglobulins were obtained from commercial sources as follows: rabbit anti-7S γ-globulin (Behringwerke); goat anti-19S γ-globulin (Hyland Laboratories, Los Angeles, Calif.); and rabbit anti-β_{2A}-globulin (Behringwerke). All of these antisera formed a single and characteristic precipitation line upon immunoelectrophoretic analysis with normal human serum. Rabbit antiserum against whole human serum (Behringwerke) was also employed.

Preparation of disrupted material from red cell membranes. Red cell membranes that were treated first with trypsin and then with autologous serum and PG were freed of their hemoglobin following the procedure of Dodge *et al*(5). Erythrocytes were subjected to osmotic lysis in 0.005 M phosphate buffer, pH 7.4, and the membranes were collected by centrifugation at 66,000 × *g* for 30 minutes. Three or 4 washings in this medium afforded the removal of hemoglobin. The membranes were then washed in isotonic veronal buffer, collected as a pellet, suspended in half their volume of veronal buffer, and subjected to sonication in a magnetostrictive oscillator for 30 minutes (200 watt, 10 kc, Raytheon Manufacturing Co., Waltham, Mass.).

Electrophoresis. The sonicated material from red cell membranes was applied to a block of Pevikon C-870 in veronal buffer, pH 8.6, ionic strength 0.05, as described previously. Electrophoresis was carried out at 4°C for 16 hours, employing 3.5 V/cm. Immunoelectrophoresis was performed according to Scheidegger(6).

Protein determination was carried out according to the method of Lowry *et al*(7).

Determination of β_{1C}, β_{1E}, 7S γ and 19S γ-globulin. The relative concentration of these proteins, in the material eluted from the Pevikon block, was ascertained by an agglutination inhibition technique. Four batches of red cells, each coated with one of the aforementioned proteins, were prepared and their agglutination patterns determined with suitable dilutions of the corresponding antisera. Then each of the electrophoretic fractions was examined for its capacity to inhibit the agglutinating effect of the antisera.

Results. Effect of polyethylene glycol on complement and γ-globulin in whole human serum. To examine the effect of PG on complement, various concentrations of PG were added to human serum, the reaction mixtures adjusted to give a 1:2 dilution of serum and incubated at 20°C for 30 minutes. The turbidity which developed was quantitated by reading the optical density at 650 mμ in a Coleman photocolorimeter. The residual hemolytic activity of the treated samples was determined on aliquots suitably diluted with veronal buffer to eliminate any effect of PG on the hemolytic reaction. The results of these determinations are presented in Fig. 1, where it can be seen that increasing concentrations of PG resulted in progressive inactivation of complement as well as in progressive development of turbidity.

To clarify the cause of the observed correlation between turbidity and inactivation of complement, the turbid material was collected by centrifugation, washed in PG containing buffer and resuspended in normal saline. Since suspension of the precipitate in PG-free solvent resulted in its instant and complete dissolution, it could readily be sub-

jected to immunoelectrophoretic analysis. Precipitation lines characteristic for 7S γ_2 and 19S β_{2M}-globulin were consistently found, as well as lines that correspond to β_{1E} and β_{1C}-globulin, 2 components of the complement system.

These results are interpreted to indicate that treatment of human serum with a criti-

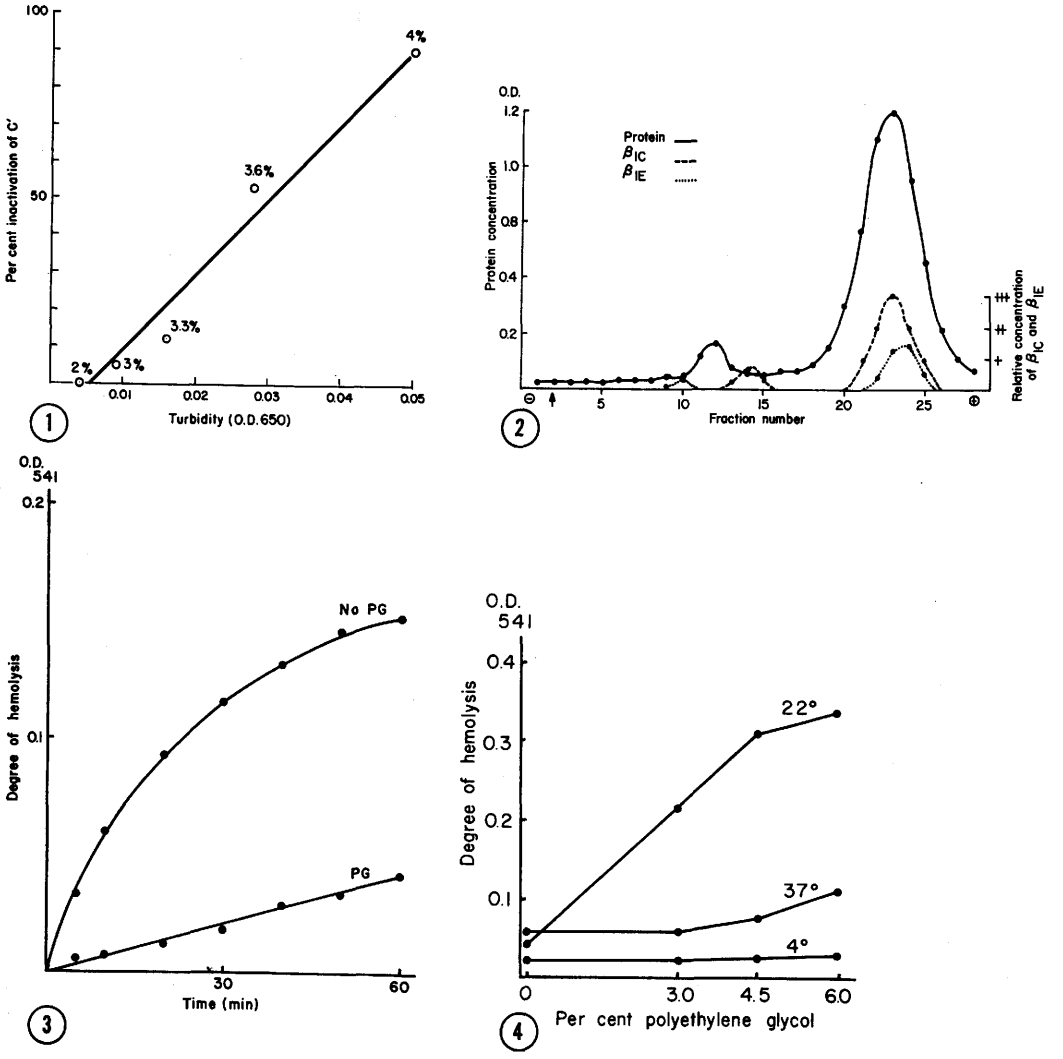

FIG. 1. Effect of treatment of human serum with various concentrations of polyethylene glycol. The linear relation between appearance of turbidity and inactivation of hemolytic complement suggests a causal connection between the 2 phenomena.

FIG. 2. Preparative zone electrophoresis on Pevikon block of sonicated complement treated red cell membranes. The major portion of β_{1C}- and β_{1E}-globulin remained closely associated during electrophoresis with the rapidly migrating material from disrupted membranes. Minor protein peak (fract. 11-13) represents hemoglobin. Arrow indicates point of application.

FIG. 3. Effect of PG on manifestation of immune hemolysis of sheep erythrocytes. Both samples of sensitized cells were treated with complement for 2 min at 37°C. Cells were then washed in cold buffer and one sample resuspended in buffer, the other in buffer containing 4.5% PG. PG markedly reduced ensuing hemolysis. However, upon removal of PG, lysis reached the values of the control sample.

FIG. 4. Effect of temperature on lysis of trypsin-treated human red cells by autologous serum in presence of various concentrations of polyethylene glycol. One hundred % lysis corresponds to an O.D. of 0.420.

TABLE I. Uptake of β_{1C} and β_{1E}-Globulin by Human Red Cells Treated with Various Enzymes and then Reacted with Autologous Serum in Presence of Polyethylene Glycol.

Treatment	No. positive reactions/ No. individuals tested
None	0/16
Trypsin	36/36
Chymotrypsin	2/4
Neuraminidase	15/21
β-Glucuronidase	0/7
Pancreatic lipase	0/4

cal concentration of PG leads to formation of reversible aggregates of γ-globulin, which in turn react with complement causing its inactivation.

Uptake of complement by human red cells treated with polyethylene glycol and autologous serum. The possibility that human red cells treated with autologous serum in the presence of PG might bind complement was explored as follows: a suspension of red cells containing PG was equilibrated at 37°C, whereupon autologous serum was added. The final concentrations of red cells, PG and serum were 1%, 4.5% and 10%, respectively. The reaction mixture was incubated at 37° for 60 minutes; thereafter the supernatant was separated from the cells for determination of degree of hemolysis. The red cells were washed twice, resuspended in veronal buffer and then tested for the presence on their surface of the complement components β_{1E} and β_{1C}-globulin. For this analysis an agglutination technique employing specific antisera was used. Results (Table I) indicate that complement components were not taken up under these conditions, and that no hemolysis occurred.

At this point it was considered necessary to investigate whether certain enzymes might condition the red cell membrane for uptake of complement upon treatment with serum and PG. The results are summarized in Table I and show that pre-treatment of the red cells with either trypsin, chymotrypsin, or neuraminidase lead to uptake of complement, as demonstrated by the agglutination of these cells with antisera against β_{1E} and β_{1C}-globulin.

That uptake of these complement components by trypsin-treated red cells is a specific manifestation of the complement reaction is shown in Table II. The phenomenon does not occur when fresh serum is replaced in the reaction mixture by either heat-inactivated or EDTA containing serum. It is also shown in Table II that none of the immunoglobulins are specifically taken up during the PG-mediated complement reaction, as demonstrated by lack of agglutination by antisera directed against 7S γ-, 19S γ- or β_{2A}-globulin. Similar results were obtained with neuraminidase-treated red cells.

To find out whether panagglutinins play any role in uptake of complement by trypsinized erythrocytes reacted with autologous serum and PG, the following experiment was carried out. Serum was subjected to multiple absorptions with trypsinized erythrocytes and was then reacted with a fresh batch of trypsinized red cells in the presence of PG. It was found that the degree of uptake of β_{1C} and β_{1E}-globulin was similar to that following the use of unabsorbed serum. It is, therefore, concluded that panagglutinins did not participate in any essential manner in the PG-mediated autologous reaction.

To test the firmness of the link between β_{1E} or β_{1C} and the cell membrane, the following experiment was carried out. Mem-

TABLE II. Specificity of Uptake of β_{1C} and β_{1E}-Globulin by Trypsin-Treated Human Red Cells Reacted with Autologous Serum in Presence of Polyethylene Glycol.

Treatment of red cells	Agglutination by Anti-β_{1C}	Anti-β_{1E}	Anti-7Sγ	Anti-19Sγ	Anti-β_{2A}
Trypsin; PG, serum	+++	+++	0	0	0
" ; " , heated serum	0	0	0	0	0
" ; " , EDTA-serum	0	0	0	0	0
Serum, PG	0	0	0	0	0
Trypsin	0	0	0	0	0
" ; serum	0	0	0	0	0

branes were prepared of cells which had previously taken up complement during treatment with PG and serum. These membranes were disrupted by sonication and were then subjected to preparative electrophoresis, employing Pevikon as supporting medium. Following electrophoresis, each segment was eluted and analyzed for protein and for β_{1E}, β_{1C}, 7S γ and 19S γ-globulin. The results are shown in Fig. 2 and indicate that the protein from the disrupted red cell membranes migrated as a single broad peak with an electrophoretic mobility somewhat slower than that of albumin. The bulk of β_{1E} and β_{1C}-globulin migrated closely associated with the main protein fraction and with a similar rapid mobility. A small proportion of β_{1E} and β_{1C}-globulin was found in the β region, where these 2 globulins are found when they are in the free state.

The presence of most of the β_{1E} and β_{1C}-globulin in the area of the rapidly migrating main protein fraction suggested that these complement components remained combined with the membrane particles produced by sonication. There was, however, an alternative explanation. β_{1E} and β_{1C}-globulin might have been dissociated from the membrane during sonication and might have undergone changes in their electrophoretic properties. However, analysis of the material from the main electrophoretic peak in a density gradient disclosed that both β_{1E} and β_{1C}-globulin sedimented with the membrane particles, *i.e.*, with a velocity many times their own sedimentation rate. It is, therefore, concluded that a large proportion of the β_{1E} and β_{1C}-globulin that was taken up by the cell membrane in the course of the reaction with PG and serum remained bound to membrane constituents even after disruption of the membranes by sonication and even during separation of the solubilized material in the electric field. That the binding of complement is independent of bound γ-globulin is again demonstrated by the failure to detect 7S γ or 19S γ-globulin in any of the electrophoretic fractions.

Lysis of human red cells treated with polyethylene glycol and autologous serum. Since human red cells pre-treated with trypsin or neuraminidase bind complement when reacted with PG and serum, hemolysis should be expected to occur. However, in many instances, no significant degrees of lysis were found. To find out whether in these cases PG prevented lysis of complement damaged cells by virtue of its colloid-osmotic pressure, the following experiment was performed. Two tubes containing the same amount of sensitized sheep cells were incubated at 37° with a 1:50 dilution of human serum for 2 minutes. The complement reaction was stopped by addition of cold veronal buffer, and after centrifugation, the cells from one tube were resuspended in veronal buffer containing 0.01 M EDTA, while those from the other tube were suspended in veronal buffer containing, in addition to 0.01 M EDTA, PG in a concentration of 4.5%. Both tubes were then incubated at 37° and aliquots were withdrawn at various intervals to determine the degree of hemolysis. As seen in Fig. 3, the presence of PG markedly interferes with the development of lysis in cells already acted upon by complement. That this inhibition is chiefly due to an interference with entrance of water into the complement treated cells was shown by resuspending the cells in cold veronal buffer devoid of PG and keeping them in the cold for 30 minutes. Under these conditions, lysis occurred and approached the level of hemolysis of the control cells which were incubated with serum in the absence of PG.

In view of the osmotic effect exerted by PG, the hemolytic effect of PG and complement in an autologous system was studied as follows. After 1 hour of incubation of trypsin-treated cells and autologous serum in the presence of PG, the reaction mixture was diluted 1:3 with cold veronal buffer. By thus lowering the PG concentration at the end of the incubation period, the true degree of hemolysis became apparent. The unlysed cells were removed and the concentration of free hemoglobin was measured. The results shown in Fig. 4 demonstrate that there is no production of hemolysis at 4°C and that at 22° the amount of lysis is significantly higher than at 37°.

To explain this rather unexpected finding

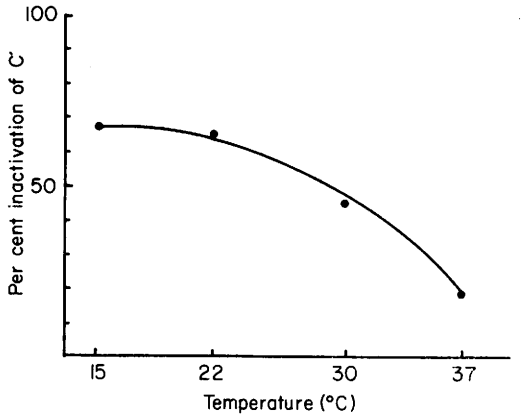

FIG. 5. Effect of temperature on inactivation of complement by treatment of human serum with 4.5% polyethylene glycol.

of a much greater degree of hemolysis at 22° than at 37°, it was attempted to relate this phenomenon to the temperature dependent efficiency of PG to induce the complement reaction. Inactivation of complement by 4.5% PG was determined at various temperatures. The results (Fig. 5) indicate that maximal inactivation of complement occurs at temperatures lower than 37°.

Discussion. Treatment of human serum with appropriate concentrations of PG leads to inactivation of complement and to formation of aggregates of γ-globulin. The close correlation between these two phenomena suggests a causal relationship between them. It is well known that one of the properties of aggregated γ-globulin is its capacity to inactivate hemolytic complement(8). It appears quite probable, therefore, that the primary effect of PG on serum is the production of reversible aggregates of γ-globulin, and that as a consequence thereof complement is inactivated. This sequence of events may also explain the function of PG as a nonspecific sensitizer in red cell-complement systems. Cowan(1) suggested that this agent might act by directly mediating the reaction of complement with the cell membrane. The data presented here indicate that PG first leads to formation of aggregates of γ-globulin, and that these aggregates then become instrumental in mediating the interaction between complement and cell membranes.

That hemolysis is more readily produced at 22°C than at 37°C is not surprising, in view of the similarly temperature dependent inactivation of complement by PG. Turbidity measurements have shown that the efficiency of a given concentration of PG to produce aggregates of γ-globulin is also greater at 20° than at 37°. The observation that PG inhibits lysis of complement damaged cells is relevant in relation to estimates of the size of the membrane lesion caused by complement. Green *et al*(9) have shown that albumin, but not sucrose, is capable of preventing lysis of complement treated cells. Thus, the lesion was considered larger than a molecule of the molecular weight of 342, but smaller than a macromolecule of the size of albumin. Since the PG employed in this study is of the average molecular weight of 3700, it may be concluded that the lesion is of a rather small size.

The primary aim of this study was to find out whether certain complement components are capable of combining directly with the cell membrane. Theoretically, an alternative possibility existed, namely that complement attaches itself to the antibody molecules which are coating sensitized cells. To determine the role of antibody in complement binding, the use of reversibly combining sensitizers was indicated, since they can be quantitatively removed from the cells following the action of complement. Polyethylene glycol was chosen because it had been thoroughly investigated by Cowan(1). The most important result of this investigation is the finding that certain complement components are taken up by the cell membrane in the absence of antibody and that these components establish a firm and permanent link with membrane receptors. Specific uptake and retention of complement is demonstrated here for β_{1E}-globulin, carrier of the classical C'4 activity(4), and β_{1C}-globulin, which possesses C'3 activity(10). Since the first component of complement can be removed from complement treated cells without affecting the cell bound C'4(11), it must be inferred from the presented data that β_{1E}-globulin attaches itself directly to membrane receptors. Whether β_{1C}-globulin also com-

bines directly with membrane sites or with sites on bound β_{1E}-globulin cannot be decided on the basis of these experiments. Evidence will be presented elsewhere, however, suggesting that β_{1C}-globulin forms a direct link with the cell membrane independent of β_{1E}-globulin (Müller-Eberhard, to be published). That permanently cell bound γ-globulin molecules are not involved in this phenomenon is indicated by the failure to agglutinate complement-coated red cells with specific antisera to immunoglobulins and by the failure to demonstrate 7S or 19S γ-globulin in the soluble material from sonicated complement containing cell membranes. In fact, one of the reasons for performing all experiments with autologous systems was to secure the absence of anti-red cell antibodies.

These results also explain the previously reported observations on red cells from certain patients with acquired hemolytic anemia (12). In some cases, such cells were found to be coated *in vivo* with both β_{1E} and β_{1C}-globulin. However, as indicated by negative Coombs tests, these cells were not coated with antibody. If anti-red cell antibody was instrumental in attaching complement to these cells, it must have dissociated from the cells later on. Harboe (personal communication) recently showed that cold agglutinins can be quantitatively eluted *in vitro* from complement-treated human red cells without removing either β_{1E} or β_{1C}-globulin. Alternatively, unrelated soluble antigen-antibody complexes might have been responsible for the observed complement uptake. This possibility is suggested by the work of Dixon(13) and that of Shulman(14).

That complement uptake and hemolysis occur only with cells previously treated with either trypsin or neuraminidase indicates an inhibitory effect of cell bound neuraminic acid. An inhibitory role of neuraminic acid in complement-red cell interaction has been suggested previously by Yachnin *et al*(15) in studies of hemolysis at acidic pH and recently by Arquilla *et al*(17). According to Springer and Rapaport(16), both trypsin and neuraminidase release neuraminic acid from erythrocytes. This negatively charged carbohydrate residue may conceivably exert a repelling effect upon the likewise negatively charged complement components. If that is so, then it must be assumed that anti-red cell antibody normally counteracts the effect of membrane bound neuraminic acid. For in the presence of specific antibody prior enzymatic treatment of cells is not required for hemolysis and complement fixation. On the other hand, the possibility exists that neuraminic acid plays a significant role in reactions not involving anti-red cell antibodies but unrelated antigen-antibody complexes or γ-globulin aggregates. Under these circumstances, complement binding to the red cell may occur only if it is preceded by an enzymatic alteration of the cell surface. Whether such a concept has validity for *in vivo* immune reactions and for cells other than erythrocytes deserves further exploration.

Summary. The molecular interaction between complement and red cells has been analyzed in autologous human systems. Instead of anti-red cell antibody, polyethylene glycol was used as a non-specific sensitizer. It was found that as a result of complement-cell membrane interaction, certain complement components form a direct and firm link with membrane receptors. Polyethylene glycol mediates the action of complement on cells apparently through reversible aggregation of γ-globulin. Cell bound neuraminic acid seems to have a protective effect against the cytolytic action of complement. In the autologous system employed, significant uptake of complement and subsequent hemolysis occurred only after pre-treatment of the red cells with trypsin or neuraminidase.

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Interferon in Dermal Crusts of Human Vaccinia Virus Vaccinations Possible Explanation of Relative Benignity of Variolation Smallpox.* (29659)

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Interferon, a protein with virus inhibitory properties synthesized in cells in response to viral infection, has been detected in a variety of cell culture and animal systems infected with vaccinia virus(1-4). Further, recent reports have revealed the presence of interferon-like substances in acute pharyngeal washings of patients with influenza(5), in human cerebrospinal fluid(6), in sera of patients with clinical viral infections(7) and in sera and organ extracts following intravenous administration of viruses to a patient with acute myelogenous leukemia(8).

This project on detection of interferon in dermal crusts of vaccinia virus vaccinations of man was conceived in an attempt to provide an explanation of the relative benignity of variolation smallpox.

Variolation, a method of vaccination against smallpox, was utilized in ancient China and is still in use in primitive areas of the world today. This method consists of the intradermal inoculation of crusts from the skin lesions of patients with smallpox. Although variolation usually produces a mild form of smallpox, occasionally severe or even fatal disease occurs.

The mechanism responsible for the mildness of variolation smallpox is unknown. However, the recent discovery of interferon suggested the possibility that this substance might be present, together with variola virus, in the crusts of smallpox skin lesions and thus influence initial virus multiplication at the vaccination site.

Since smallpox dermal crusts were not available for the present investigation, crusts from vaccinia virus vaccinations were examined for interferon. In this communication the detection of interferon in 4 of 5 vaccinia vaccination crusts is reported and the possible role of interferon in limiting the spread of vaccinia virus in vaccinations and in attenuating the clinical course of variolation smallpox is discussed.

Materials and methods. Dermal crusts from vaccinia virus vaccinations were ground to

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