INFLUENCE OF ERYTHROCYTE MEMBRANE COMPONENTS ON MALARIA MEROZOITE INVASION

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(Received for publication 5 September 1973)

Plasmodium species are characterized by their limited host range. Infections by some plasmodia are even limited to a subpopulation within one species (e.g., resistance of blacks to Plasmodium vivax) (1) or to a specific age group of erythrocytes (e.g., reticulocyte preference of P. vivax, P. cynomolgi, and P. berghei) (2). Experimental evidence that host specificity is determined, in some cases, by the outer surface of the erythrocyte membrane was first presented by McGhee (3, 4). He found that merozoites of P. lophurae, an avian malaria that infects both chick embryo and duck erythrocytes, had a much higher affinity for duck erythrocytes. Recently, Butcher et al. demonstrated that in vivo infectivity of P. knowlesi for various host species can be related in vitro to the penetration of erythrocytes of these species (5).

We have also observed that P. knowlesi merozoites invaded erythrocytes of species that are susceptible in vivo (rhesus monkey [Macaca mulatta], man, and night monkey [Aotus trivirgatus]). (The methods were the same as described in this study.) However, rhesus erythrocytes were infected by four times more merozoites than human erythrocytes (eight experiments) or A. trivirgatus erythrocytes (three experiments) under identical conditions. Invasion of erythrocytes in vitro was rare in resistant species (mouse, rat, hamster, chicken, chick embryo, dog, and rabbit). All these studies suggest that host range may be determined by specific membrane components on the outer surface of the erythrocyte membrane. The effects of enzymatic treatment of human and rhesus erythrocytes on the penetration by P. knowlesi merozoites were studied to define the chemical nature of the erythrocyte membrane receptor.

Materials and Methods

Heparinized blood was drawn from P. knowlesi infected rhesus monkeys when the parasitemia was greater than 8% and the majority of schizonts had four or more nuclei. Platelets were removed by the addition of ADP (1 mg ADP/ml blood), followed after 2 min by filtration over glass beads (0.012-0.11 mm). After removal of plasma by centrifugation, the cells
were washed once with culture medium (Medium 199 plus 10 mM glucose, 6.6 mM glycyl-glycine, 10% heat-inactivated fetal calf serum, 50 U of penicillin, and 50 μg streptomycin per ml). Washed cells were placed in microhematocrit tubes and centrifuged for 1.25 min. The low density, infected erythrocytes formed a brown layer above the uninfected erythrocytes. The tubes were scored and broken at the upper part of the brown layer, and the schizonts were added to fresh medium. This cell suspension that consisted of 99% schizont-infected erythrocytes was diluted to 1,000 cells/mm³.

Normal human and rhesus erythrocytes were treated with various enzymes. The plasma and buffy coat were removed from heparinized blood, and the erythrocytes were washed three times in Tris total buffer (6), pH 7.5, (for chymotrypsin, Pronase, and trypsin) or phosphate-buffered saline (PBS), pH 7.4, (for neuraminidase). Erythrocytes suspended in their respective buffers (100,000 cells/mm³ for chymotrypsin, trypsin, and neuraminidase or 500,000 cells/mm³ for Pronase) were exposed to enzymes at 37°C for 20 min (trypsin 2,210 U/ml), 90 min (trypsin 44 and 4.4 U/ml, chymotrypsin and Pronase) or 150 min (neuraminidase). The sources of enzymes were as follows: neuraminidase (Clostridium perfringens), type VI, Sigma Chemical Co. (St. Louis, Mo.); trypsin, TRL, Worthington Biochemical Corp. (Freehold, N. J.); Pronase, B grade, Calbiochem (San Diego, Calif.); chymotrypsin, CDS, Worthington. The control erythrocytes were handled in the same manner except for the absence of enzyme during the 37°C incubation.

Normal or enzyme-treated human or rhesus erythrocytes were suspended in culture medium at a concentration of 10,000 cells/mm³ and combined in equal volumes with parasitized erythrocytes. This mixture was inoculated into a Dvorak-Stotler culture chamber (7) maintained at 35°C and continuously perfused with fresh culture medium at a rate of about 0.44 ml/h. Merozoites were observed to rupture from infected erythrocytes and invade susceptible erythrocytes. After about 4 h the cells were removed from the chamber, spread in a thin film on slides, and Giemsa stained. The percent of erythrocytes invaded by merozoites was determined from these preparations by counting until either 50 infected erythrocytes or 5,000 uninfected erythrocytes had been observed. Untreated cells were included as a control in each experimental series.

Erythrocyte membrane components were added to the culture medium in an attempt to inhibit invasion of normal rhesus or human erythrocytes. (a) Normal human erythrocyte sialoglycoprotein isolated by the method of Marchesi and Andrew (8) was suspended in the perfusion medium at a concentration of 100-125 μg/ml. (b) Normal human erythrocytes (500,000 cells/mm³) were treated with Pronase (22.5 U/ml) in Tris total buffer for 2 and 4 h at 37°C. The cells were removed by centrifugation and the supernate was placed in boiling water for 4 min (9). A 1:50 dilution of the heated supernatant fluid was placed in culture medium.

The surface charge of enzyme-treated and normal erythrocytes was measured as electrophoretic mobility at 23°C in a Zeiss cytopherometer (Carl Zeiss Inc., New York) using a low ionic strength phosphate-sorbitol buffer, pH 7.2 (10). At least 10 cells in each sample were measured, with a second measurement after the reversal of polarity. The average electrophoretic mobility for normal human erythrocytes was 2.14 μm/s/V/cm.

RESULTS

Treatment of human erythrocytes with chymotrypsin or Pronase almost eliminated invasion by merozoites, whereas invasion was not inhibited by either neuraminidase or trypsin (Table I). Reduction in electrophoretic mobility was much greater in neuraminidase-treated erythrocytes (87%) than in chymotrypsin-treated erythrocytes (21%) (Table I). This clearly indicates that negative surface charge on the erythrocyte is unrelated to susceptibility to invasion.

Rhesus erythrocytes, treated with neuraminidase, neuraminidase followed
TABLE I

Effect of Enzyme Treatment of Human Erythrocytes on Invasion by Plasmodium knowlesi Merozoites and on Electrophoretic Mobility

| Enzyme (concn) | Infected RBC* per 10,000 RBC | Ratio of treated: control | Electrophoretic mobility (% Reduction) |
|----------------|-----------------------------|--------------------------|--------------------------------------|
|                | Treated                      | Control                  |                                       |
| Chymotrypsin   |                             |                          |                                       |
| (47 U/ml)      | 0                            | 40                       | 0.0                                  | 18                     |
| (47 U/ml)      | 1                            | 20                       | 0.05                                 | 21                     |
| (4.7 U/ml)     | 2                            | 40                       | 0.05                                 | 11                     |
| Pronase        |                             |                          |                                       |
| (225 U/ml)     | 0                            | 180                      | 0.0                                  | 53                     |
| (22.5 U/ml)    | 4                            | 40                       | 0.1                                  | 61                     |
| (22.5 U/ml)    | 0                            | 110                      | 0.0                                  | 64                     |
| (2.25 U/ml)    | 0                            | 40                       | 0.0                                  | 37                     |
| (2.25 U/ml)    | 10                           | 60                       | 0.2                                  | 39                     |
| Neuraminidase  |                             |                          |                                       |
| (0.1 U/ml)     | 150                          | 190                      | 0.8                                  | 85                     |
| (0.1 U/ml)     | 310                          | 110                      | 2.8                                  | 87                     |
| Trypsin        |                             |                          |                                       |
| (2210 U/ml)    | 220                          | 110                      | 2.0                                  | 25                     |
| (44 U/ml)      | 30                           | 40                       | 0.8                                  | 29                     |
| (4.4 U/ml)     | 110                          | 40                       | 2.8                                  | 17                     |

* The counts were the sum of duplicate chambers. At high rates of invasion (>100 infected RBC per 10,000 RBC), less than 10,000 RBC were counted and the numbers in the table estimated.

by periodation (1 mM for 10 min), trypsin, chymotrypsin, or Pronase, were as susceptible to invasion as untreated erythrocytes. Treatment of rhesus erythrocytes with concentrations of enzymes comparable to those used in treatment of human erythrocytes had less effect on electrophoretic mobility of rhesus erythrocytes; chymotrypsin had no effect on electrophoretic mobility.

Attempts at blocking invasion by the addition of erythrocyte membrane components to the culture medium were unsuccessful. In six experiments erythrocyte sialoglycoprotein did not inhibit invasion of normal human or monkey erythrocytes. Pronase digests from human erythrocytes also had no influence on invasion of normal human erythrocytes.

**DISCUSSION**

Variation in susceptibility of erythrocytes from different species to invasion by merozoites of *Plasmodium* species suggests the presence of a specific receptor on the erythrocyte surface (references 3–5 and present paper). The fact that chymotrypsin- and pronase-treated human erythrocytes were refractory to invasion by *P. knowlesi* merozoites supports this hypothesis and indicates that the receptor is a protein or lipoprotein complex. Susceptibility of this erythrocyte receptor to chymotrypsin and Pronase and its resistance to trypsin should provide leads as to its structure.
One major protein component of the erythrocyte surface, variously named 3 (11), a (12), or E (13), is cleaved by chymotrypsin and Pronase but unaffected by trypsin (11). Protein E, unlike the other major protein component of the outer surface, the sialoglycoprotein, lacks sialic acid and contains only 7% carbohydrate as compared to 54% for sialoglycoprotein (13). This protein (3, a, or E) spans the erythrocyte membrane from the outer surface to the interior of the cell (11, 12). If this protein is a receptor, it can be postulated that the receptor itself also is instrumental in the penetration of the parasite into the host erythrocyte. Obviously other surface components could have the same pattern of susceptibility to Pronase and chymotrypsin and resistance to trypsin. Preliminary data suggest that one such component, erythrocyte acetylcholinesterase, is not the receptor.

Trypsin, chymotrypsin, and Pronase cleave sialoglycoproteins from human erythrocyte membranes (6, 14, 15). However, treatment with trypsin results in greater release of sialic acid and greater reduction in electrophoretic mobility than chymotrypsin (14). In both cases, release of sialic acid by these proteolytic enzymes is due to cleavage of sialoglycopeptides from sialoglycoprotein. Since trypsin treatment did not inhibit penetration and chymotrypsin treatment did, sialoglycoprotein is probably not the receptor in this system. This is supported by the failure to inhibit invasion by removal of sialic acid from erythrocyte sialoglycoprotein by neuraminidase or by adding human erythrocyte sialoglycoprotein to the culture medium. Absence of a neuraminidase effect confirms previous reports that neuraminidase treatment of duck erythrocytes did not influence invasion by *P. lophurae* (4, 16).

Invasion of monkey erythrocytes was unaffected by pretreatment with Pronase, chymotrypsin, trypsin, or neuraminidase. Moreover, chymotrypsin treatment that reduced electrophoretic mobility of human erythrocytes had no effect on monkey erythrocytes. That the effect of enzymes on the outer surface of the erythrocyte membrane varies for different species is well documented (14). Differences among species may be explained by the amino acid content of proteins, inaccessibility of some proteins to enzyme action or the adsorption of enzymes to some membrane surfaces. Alternately, the high affinity between *P. knowlesi* merozoites and monkey erythrocytes may require greater alteration of the receptor to inhibit invasion.

One theoretical approach to the control of malaria is immunization of susceptible hosts. The identification of the receptor on erythrocyte membranes might permit isolation of the component on the merozoite that initiates invasion. It may then be possible to immunize the host against this site on the merozoite in order to block erythrocyte penetration.

**SUMMARY**

Chymotrypsin- and Pronase-treated human erythrocytes were refractory to invasion by *P. knowlesi* merozoites; invasion was not inhibited by trypsin or
neuraminidase treatment. These data implicate a surface protein other than sialoglycoprotein as the receptor site for merozoites. Invasion of rhesus erythrocytes was unaffected by pretreatment with these enzymes. Differences in membrane structure of erythrocytes from various species may explain the absence of an enzyme effect on rhesus erythrocytes.

We thank Dr. B. Hourani, National Institutes of Health, for supplying erythrocyte sialoglycoprotein, and Dr. G. Pacheco for suggestions on the manuscript.

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