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EVIDENCE FOR A TEMPERATURE-DEPENDENT AND TEMPERATURE-INDEPENDENT PATHWAY IN THE GENERATION OF COMPLEMENT-MEDIATED TRANSMEMBRANE CHANNELS

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SUMMARY: The formation of transmembrane channels by the ninth component of complement in sheep red cell membranes is the result of a series of reactions that occur in and on the membrane after the binding of this component. The energy requirements of this series of reactions vary with the species of the eighth component of complement to which the ninth component is bound. Furthermore, the results provide the first evidence that two mechanisms of channel formation exist, one that is temperature-independent and the other that has the characteristics of an enzymatic reaction with a Q10 close to 2.

INTRODUCTION

Lysis of sheep erythrocytes (E)[1] by antibody and complement (C) has been shown to occur following the generation of a transmembrane channel and disruption of the semipermeable properties of the cell (1-4). The generation of the transmembrane channel after the binding of C9 to EAC1-8^{hu} requires several steps, some of which are influenced by temperature and at least one of which is inhibitable by metal salts (5). These observations suggest that activation or an energy requiring rearrangement of at least a portion of the C5-9 complex alone or possibly in conjunction with the cell membrane occurred during the generation of the transmembrane channel.

The experiments in this study were designed to examine how temperature affects the reaction EAC1-$9^{bound} \rightarrow$ EAC1-$9^{inserted}$ and the reaction EAC1-$9^{inserted} \rightarrow$ EAC1-9^{doomed} (the latter reaction inhibitable by metal salts).

[1] E, sheep red cell; A, rabbit IgM anti-Forssman antibody; C, complement; Hu or hu, human; GP or gp, guinea pig; compound abbreviations are derived from these elements, e.g., EAC1-8^{hu} represents sheep red cells sensitized with rabbit IgM anti-Forssman antibody and carrying human complement components C1 through C8 on their surface; VBS-gel, Veronal-buffered saline containing 0.1% gelatin (6).

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The results indicated that the energy requirements are not the same for guinea pig and human C8 and C9 and revealed additional complexities in the generation of the transmembrane channel.

MATERIALS AND METHODS

Cells. E were collected and washed as described in (6).

Buffers. All buffers were prepared as described in (6).

Preparation of EAC1-7. EAC1-7 were prepared as described in (4).

Preparation of EAC1-9^{bound} and EAC1-9^{inserted} intermediates. The EAC1-8^{hu}9$^{hu\ bound}$ cells were prepared by incubating EAC1-8^{hu} (2×10^9/ml) (prepared with excess C8) with an equal volume of 1/20 dilution of HuC9 for 15 min at 0°C in VBS-gel ($\mu = 0.15$). The unbound C9 was removed by washing with ice-cold VBS-gel. EAC1-9$^{hu\ inserted}$ were prepared by incubating these EAC1-9$^{hu\ bound}$ in 5×10^{-4} M $ZnSO_4$ for 30 min at 37°C. Consequently the EAC1-9^{bound} and EAC1-9^{inserted} intermediates differ only in the stage in the lytic reaction sequence but have identical distribution of C9 and other C components. The corresponding intermediates with GPC8 and GPC9 were prepared in the same way except GP components were substituted for the appropriate human components.

Measurement of the kinetics of lysis of EAC1-9^{bound} or EAC1-9^{inserted}. The kinetic experiments were carried out by adding aliquots of EAC1-9^{bound} or inserted intermediates to tubes containing 1 ml of VBS-gel preincubated at 0°, 10°, 23° and 37°C and incubating at these temperatures for varying times before determining the degree of hemolysis. Since it was not possible to start the reactions at four different temperatures simultaneously, the zero time points at each temperature were staggered over a period of approximately 3 min. The reactions were started in the order 0°, 10°, 23° and 37°C, respectively. Under these conditions small differences were observed in the degree of lysis at time zero.

RESULTS AND DISCUSSION

There are at least three steps in the lysis of EAC1-9^{bound} prepared with HuC8 and HuC9: EAC1-9^{bound} → EAC1-9^{inserted} → EAC1-9^{doomed} → Eghosts. These reactions may be affected in varying degrees by changes in temperature, e.g., the insertion reaction which has been shown not to occur at 0°C (3, 4), while the colloid osmotic lytic reaction, EAC1-9^{doomed} to Eghosts was found to be independent of temperature (7, 8). By comparing the effect of temperature on the conversion of EAC1-9^{bound}, EAC1-9^{inserted} or EAC1-9^{doomed} to Eghosts, it is possible to obtain information on the effect of temperature on each individual step of the reaction sequence. In the next series of experiments the kinetics of the lytic reaction of EAC1-9^{bound} or EAC1-9^{inserted} to Eghosts

was studied at 0^{o}, 10^{o}, 23^{o} and 37^{o}C using intermediates prepared with HuC8 and HuC9. The kinetics of lysis of EAC1-$8^{hu}9^{hu}$ bound shown in Figure 1A indicated that there was a lag in the reaction or reactions leading to lysis at 23^{o}C or 37^{o}C and that no detectable lysis occurred at 10^{o}C or below. In marked contrast, EAC1-$9^{inserted}$ (Fig. 1B) lysed at all temperatures with no lag. The maximal rate of generation of lytic sites on EAC1-9^{hu} inserted at 0^{o}, 10^{o}, 23^{o} and 37^{o}C was approximately 0.28, 0.33, 0.4 and 0.79 lytic sites/hr, respectively. The rate changes at 10^{o}C and above were consistent with a reaction having a Q10 of approximately 2; however, considerably more lysis occurred at 0^{o}C than would be predicted for such a reaction.

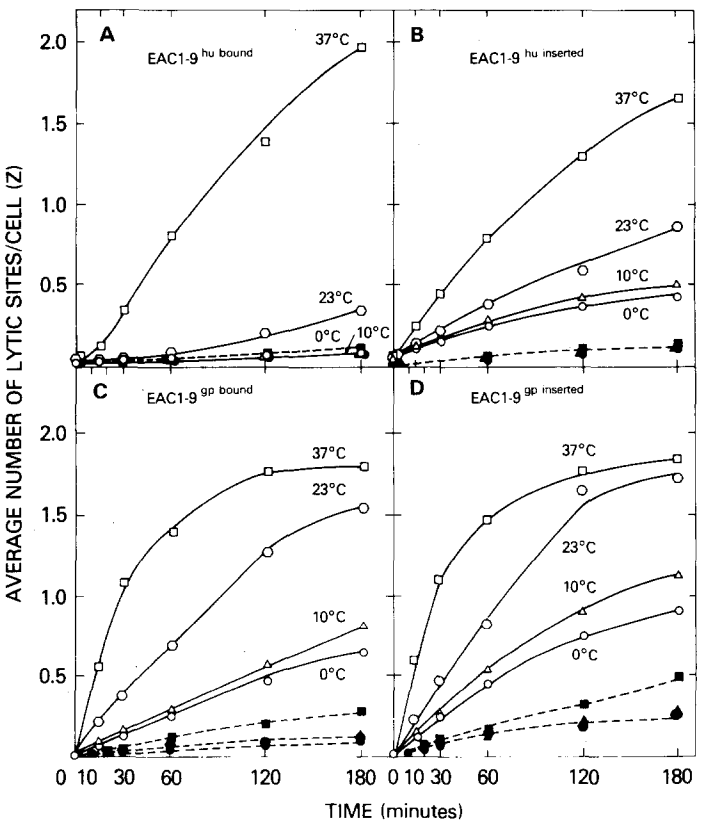

Fig. 1. Kinetics of lytic site generation (z) at 0^{o}, 10^{o}, 23^{o} and 37^{o}C of A, EAC1-$8^{hu}9^{hu}$ bound; B, EAC1-$8^{hu}9^{hu}$ inserted; C, EAC1-$8^{gp}9^{gp}$ bound; D, EAC1-$8^{gp}9^{gp}$ inserted. The solid symbols represent the results obtained with the corresponding EAC1-8 intermediates.

When these experiments were repeated using EAC1-9^{bound} or inserted intermediates prepared with GPC8 and GPC9, the results shown in Figures 1C and 1D were obtained. Lysis of EAC1-9$^{gp\ bound}$ (Fig. 1C) or EAC1-9$^{gp\ inserted}$ (Fig. 1D) occurred without a marked lag at all the temperatures tested, indicating the reaction EAC1-9^{bound} $\rightarrow$ EAC1-9^{inserted} could occur at or below 10^{o}C. This was in marked contrast to the results with intermediates containing HuC8 and HuC9 (Fig. 1A). This difference depended on the species of C8 used, i.e., when EAC1-8^{hu}9^{gp} were used, results similar to those obtained in Figure 1A were obtained while the EAC1-7^{hu}8^{gp}9^{hu} combination behaved in a similar way to the EAC1-8^{gp}9^{gp} intermediates shown in Figure 1C. It is not clear how the species of C8 influences the reaction to EAC1-9^{bound} $\rightarrow$ EAC1-9^{inserted}. Experiments in which C8^{gp} was added to EAC1-9^{bound} intermediates and vice versa suggested that the differences were not related to differences in fluidity of the erythrocyte membrane when different species of C8 were bound. It is possible that GPC8 and HuC8 bind to different sites although there is no evidence for such an idea. Alternatively the mechanism

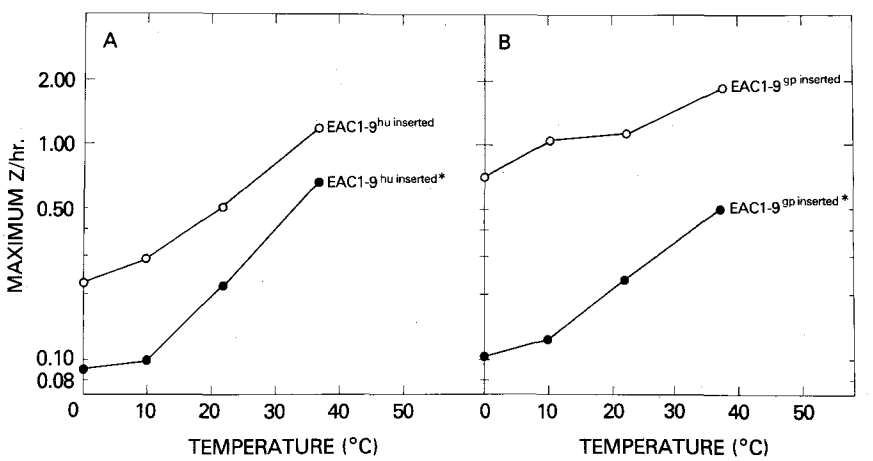

Fig. 2. Maximum rate of lytic site generation vs. temperature for A, EAC1-9$^{hu\ inserted}$ or EAC1-9$^{hu\ inserted}$ preincubated for 2 hr at 0^{o}C to remove cold sensitive population (EAC1-9$^{hu\ inserted*}$); B, EAC1-9$^{gp\ inserted}$ or EAC1-9$^{gp\ inserted}$ preincubated for 2 hr at 0^{o}C to remove cold sensitive population (EAC1-9$^{gp\ inserted*}$).

of lysis mediated by the two species of C8 may be distinct. Further characterization of the reaction of $EAC1\text{-}9^{bound} \rightarrow EAC1\text{-}9^{inserted}$ will be needed to resolve these questions.

The reaction of $EAC1\text{-}89^{hu\ inserted}$ to ghosts showed little dependence on temperature below $10^{o}C$ but above $10^{o}C$ the rate of the reaction showed a Q10 of close to 2 (Fig. 2A). When $EAC1\text{-}89^{gp\ inserted}$ were used, the temperature dependence was not as pronounced (Fig. 2B). However, for either $EAC1\text{-}9^{hu\ inserted}$ or $EAC1\text{-}9^{gp\ inserted}$ the rate of lysis at $0^{o}C$ was faster than would be expected from extrapolation of the maximal rates at higher temperatures to $0^{o}C$. These observations raised the possibility that more than one pathway of generating lytic sites might exist and that one mechanism might not be temperature dependent. In the next series of experiments the kinetics of lysis at various temperatures of populations of $EAC1\text{-}9^{gp\ inserted}$ or $EAC1\text{-}9^{hu\ inserted}$ from which most cells that will lyse at $0^{o}C$ have been eliminated by incubation at $0^{o}C$ for 2 hr were compared to the same populations without the incubation at $0^{o}C$ and the results have been plotted as the maximum rate of generation of lytic sites vs. temperature on a semi-log basis ($EAC1\text{-}9^{hu\ inserted}$ in Figure 2A and $EAC1\text{-}9^{gp\ inserted}$ in Figure 2B). It is seen that after preincubation at $0^{o}C$ for 2 hr, the Q10 for both types of cells shifted close to 2, the shift being more marked for $EAC1\text{-}9^{gp}$ than for $EAC1\text{-}9^{hu}$. The results presented in Figure 2 would be consistent with the suggestion that two mechanisms of C damage could occur: a temperature-independent reaction that occurred at $0^{o}C$ and a reaction or reactions which were temperature dependent. It is not possible to determine whether this latter reaction constitutes a single reaction with a Q10 of 2 and that the deviations observed at $0^{o}C$ in Figure 2 represent failure to correct for the contribution of the temperature-independent reaction.

From the results presented in this paper, the original scheme (3) outlined in the first paragraph of this section has to be modified to reflect the difference between GPC8 and HuC8 as well as to distinguish the possibility of

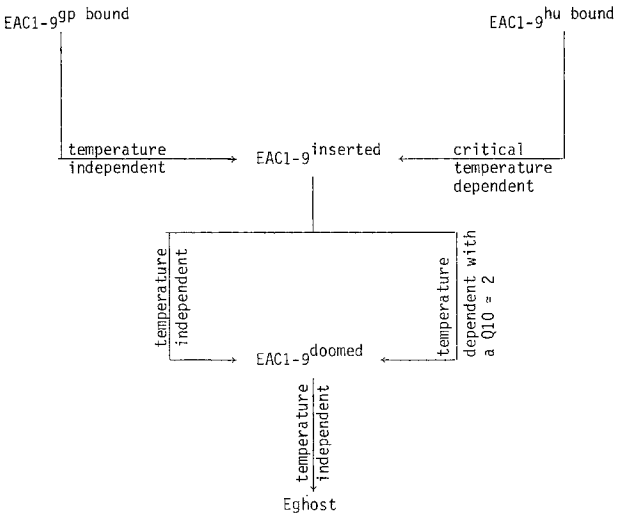

Fig. 3. Summary of the temperature-dependent steps in the reaction of EAC1-9gp bound or EAC1-9hu bound to ghosts.

more than one pathway leading to cell lysis. Consequently the scheme outlined in Figure 3 is proposed. While this scheme gives no insight into the biochemical events and physical interactions of the C components and the cell membrane leading to the formation of the transmembrane channel, it does allow for the recognition of different steps where properties can be studied separately. In particular the reaction of EAC1-$9^{inserted}$ to E^{doomed} with a Q10 of approximately 2 would be consistent with an enzymatic step, a hitherto unrecognized possibility. A search for the hypothetical enzyme and its substrate would be of importance in our understanding of the relationship of immunochemical properties to the functional activity of the C attack complex. By using targets of differing membrane composition, the influence the target (or substrate?) may have on the C attack complex may become apparent.

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