The Effect of Divalent Cations on the Rosette Formation via C3 Receptors

TOMOKO OKUDA, SATORU SATO and TAKEHIKO TACHIBANA

Department of Immunology, Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai 980

OKUDA, T., SATO, S. and TACHIBANA, T. *The Effect of Divalent Cations on the Rosette Formation via C3 Receptors.* Tohoku J. exp. Med., 1984, **143** (2), 221-229 — The indicator cells with the specificity for the various C3 receptors were prepared using highly purified complement components. The effect of EDTA and divalent cations on the rosette formation of these indicators with human blood cells and lymphoblastoid cells were investigated. B lymphocytes form rosettes with EACI-3b, EACI-3bH, EACI-3bi and EACI-3d irrespective of the presence of cations. PMN and monocytes form rosettes with EACI-3b and EACI-3bH independent of cations, but their formation with EACI-3bi needs the presence of magnesium. In this case calcium shows a cooperative effect with magnesium. PMN and monocytes do not react with EACI-3d even in the presence of magnesium. Although Raji cells do not react with EACI-3b, they react with EACI-3bH independent of divalent cations. EAC^m has a similar reactivity to EACI-3bi, and by the treatment with trypsin it becomes similar to EACI-3d. — C3 receptor; divalent cations; rosette formation; monocyte and PMN

The receptors for various C3 fragments have been detected on many types of cells — B lymphocytes, leukocytes, platelets, fibroblasts and also on some tissues. The rosette formation of cells with indicators carrying C3 fragments can be used as cell surface markers. Lay and Nussenzweig (1968) reported that EAC made from mouse serum formed rosettes with mouse peritoneal macrophages and PMN. These rosette formations depend on divalent cations and can be reversed by EDTA, while adherence to mouse lymphocytes is independent of these ions and occurs even in the presence of 0.01 M EDTA. The same phenomenon was observed and analyzed in our laboratory (Watanabe 1979). Michlmayer and Huber (1970) reported that the rosette formation of human lymphocytes with EAC^h was

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Abbreviations used in this paper: E_N, sheep erythrocytes treated with neuraminidase; EA, sheep erythrocytes sensitized with rabbit IgM fraction of anti-sheep erythrocytes; EACI-3, EA binding purified complement components from C1 to C3; EAC^m and EAC^h, EA reacted with fresh mouse or human serum respectively; PMN, polymorphonuclear leukocytes; H, factor H; I, C3b inactivator; PBS, phosphate-buffered saline; VBS, Veronal-buffered saline; GVB⁺, gelatin-Veronal-buffered saline with cations.

not inhibited by EDTA, while that of human monocytes was inhibited. Henson (1969) investigated the rosette formation of PMN from many species with EAC prepared from the isologous complement. While the rosette formation of rabbit neutrophil is inhibited by EDTA, that of human neutrophil is not. Ross et al. (1973) demonstrated that complement receptor of human B lymphocyte and that of monocyte can be distinguished, if the EAC are prepared with serum from C5-deficient mice, because human monocyte does not form rosettes in the presence of 0.04 M EDTA.

Recent development on the C3 structure showed that indicators carrying C3b, C3bH, C3bi and C3d formed rosettes with various blood cells to various degrees. In these situations it becomes necessary to reinvestigate what kind of ligand reacts with cells in the presence or absence of divalent cations using indicator cells prepared with purified complement components.

In this report it is shown that human granulocytes and monocytes form rosettes with EACl-3bi in the presence of divalent cations, although all other rosettes are formed independent of cations. The characterization of EAC^h and EAC^m, which are made from whole human and mouse sera as the complement source respectively, are also made from these results.

MATERIALS AND METHODS

Buffers and chemicals

Isotonic Veronal-buffered saline, pH 7.4, ionic strength of 0.145, containing 0.1% gelatin, 1.5×10^{-4} M CaCl₂ and 5×10^{-4} M MgCl₂ (GVB⁺⁺) and EDTA-GVB, containing a final concentration of either 0.01 M or 0.04 M EDTA were prepared according to the usual procedure (Williams and Chase 1977). Trypsin from bovine pancreas (Type III) and trypsin inhibitor from soybean were purchased from Sigma Chemical Co., USA.

Complement components and reagents

Guinea pig C1 (Nelson et al. 1966), human C2 (Vroon et al. 1970), C3 (Tack and Prah1 1976), H and I (Fearon 1977) were purified according to the published methods. Conglutinin was obtained from bovine serum according to the method of Lachmann (1973).

Indicator cells

EAC^h, EAC^m and EAC1423b (EACl-3b) were prepared using guinea pig C1 and human C4, ^{oxy}C2 and C3 as described previously (Okuda and Tachibana 1980). Briefly, EAC^h was prepared by incubating EA with highly diluted human serum at 37°C for 15 min, and EAC^m was obtained by incubating EA with higher concentration of mouse serum at 37°C for 2 hr. EACl-3bH were formed by incubating 1×10^8 EACl-3b in GVB⁺⁺ with an equal volume of H (37°C, 30 min). A preliminary investigation was carried out in order to find an optimal concentration of H to generate enough rosette forming ability with Raji cells. EACl-3bi was obtained by incubation of EACl-3b with previously determined enough amount of H and I at 37°C for 60 min. EACl-3d was generated by incubating 1×10^8 EACl-3bi in VBS with 0.1–1.0 mg trypsin (30 min, 37°C). The reaction was stopped by the addition of an excess amount of soybean trypsin inhibitor. These indicator cells were washed and suspended in GVB⁺⁺ or appropriate buffers to 2×10^8 /ml.

Cell fractionation

Human blood was withdrawn from a healthy donor and immediately mixed with one tenth volume of 0.1 M EDTA (pH 7.4), and layered on Ficoll-Isopaque and centrifuged. The interface mononuclear cell fraction was washed and seeded in plastic Petri dish pretreated with FCS, incubated at 37°C for 60 min. The adherent cells were used as the monocyte preparation, of which purity was more than 90%. The non-adherent cells were recovered, from which the rosette forming cells with neuraminidase-treated sheep erythrocytes (E_N) were removed by density centrifugation, and B lymphocytes were obtained. They contained 2% of E_N -rosette forming cells and 3% of peroxidase positive cells. The purity was estimated to be more than 90%. Polymorphonuclear leukocytes sedimented at the bottom of the Ficoll-Isopaque centrifuge tube were separated from contaminating erythrocytes by applying onto 4% Dextran T40 (Pharmacia Chemicals) in PBS. PMN was recovered from the supernate and washed with PBS and suspended to 1×10^6 /ml. They contained 88% of neutrophils, 11% of eosinophils and 1% of lymphocytes. Raji and Daudi lymphoblastoid cells (derived from Burkitt lymphoma) were grown in RPMI 1640, supplemented with 10% FCS.

Detection of C3 receptors

The rosette formation of various cells with indicators were evaluated by the published microtest plate method (Tachibana and Ishikawa 1973). Briefly, $1 \mu\text{l}$ of cells (1×10^6 /ml) in PBS was added to the well of Falcon plastic microtest plate (#3034) pretreated with poly-L-lysine. After standing at room temperature for 15 min, $10 \mu\text{l}$ of FCS was added. The plate was allowed to stand for 30 min, and washed with PBS three times. Ten microliters of indicator cells in GVB⁺⁺ or EDTA-GVB (2×10^8 /ml) were added, and reacted at 37°C for 1 hr and the plate was inverted and allowed to stand for 30 min. Unreacted indicators were detached from the bottom and the cells surrounded by four or more indicators were counted as positive cells. Six to nine hundred cells were counted and the percentage of positive cells was calculated.

Conglutination

Conglutinin-dependent hemagglutination was performed in microtiter plates containing 2.5×10^8 /ml indicator cells and serial dilutions of bovine congutinin in GVB⁺⁺. To check the specificity of congutination the same procedure was done in the presence of 0.02 M EDTA.

RESULTS

Identification of indicator cells

Table 1 defines the characteristics of indicator cells in rosette formation with

TABLE 1. *The reactivity of EAC intermediate cells*

Reaction with	Intermediate cells			
	EACI-3b	EACI-3bH	EACI-3bi	EACI-3d
Human erythrocytes	95%	75	0	0
Raji cells	0	51	54	62
Daudi cells	0	0	56	80
Conglutinin	—	—	+	—

Numbers indicate percentage of rosette forming cells.

+ or — means positive or negative congutination reaction.

TABLE 2. *Effect of EDTA on EAC-rosette formation of cells from human peripheral blood*

Indicator cells	EACI-3b		EACI-3bH		EACI-3bi		EACI-3d	
EDTA, mM	0	10	0	10	0	10	0	10
B lymphocytes	90	90	89	88	87	85	66	65
PMN	97	97	92	92	91	1	3	1
Monocytes	82	84	65	72	83	2	0	1
Erythrocytes	95	96	75	74	4	5	3	3
Raji cells	1	1	51	55	54	67	62	74

Numbers indicate percentage of positive cells.

various cells. EACI-3b was capable of producing positive immune adherence reaction with only human erythrocytes. EACI-3bH formed rosettes with human erythrocytes and Raji cells, but not with Daudi cells. EACI-3bi was lacking in reactivity with human erythrocytes, but exhibited the unique ability to be agglutinated by purified bovine congenitinin in the presence of calcium ions. EACI-3d lacked in the reactivity with human erythrocytes and congenitinin, but had high reactivity with Raji or Daudi cells. Thus it was ascertained that four types of EAC indicator cells prepared in this study had distinct reactivities from

Fig. 1. The effect of divalent cations on the rosette formation of PMN. a, b, c, rosettes with EACI-3bi; d, e, rosettes with EACI-3d.

each other.

The effect of EDTA on the rosette formation through C3 receptors

The percentage of rosette forming cells with various indicators in the presence or absence of divalent cations is shown in Table 2, which represents a typical example of several experiments giving similar results. B lymphocytes formed rosettes with all 4 kinds of indicators irrespective of the presence or absence of 0.01 M EDTA. PMN could form rosette with EACI-3bH independent of the presence of divalent cations, but their rosette formation with EACI-3bi was remarkably diminished in the absence of cations. This was the same in the case of monocytes. Raji cells did not form rosettes with EACI-3b, but invariably formed with EACI-3bH, EACI-3bi and EACI-3d even in the presence of 0.04 M EDTA.

Effect of Mg^{++} and Ca^{++} on the rosette formation of PMN or monocytes

Watanabe (1979) recognized a remarkable effect of divalent cations on the rosette formation of PMN or monocytes with EAC^m. We inquired how these cations affect the rosette formation. Monocyte or PMN suspensions in VBS which do not contain any divalent cations were mixed with indicator cells in VBS and increasing amounts of Ca^{++} or Mg^{++} were added. As can be seen from Figs. 1 and 2, Ca^{++} was not capable of inducing rosette formation of either PMN or monocytes

Fig. 2. The effect of divalent cations on the rosette formation of monocytes. a, b, c, rosettes with EACI-3bi; d, e, rosettes with EACI-3d.

with EACI-3bi, while Mg^{++} increased the number of rosette positive cells dose-dependently. The next experiment showed a synergistic effect of Ca^{++} and Mg^{++} . When one millimole of Mg^{++} equivalent to the physiological level in serum and an increasing amount of Ca^{++} were added, the percentage of rosette forming cells increased above the level of the same concentration of Mg^{++} . The rosette of PMN or monocytes with EACI-3d was not detected in the presence of any concentration of Mg^{++} or Ca^{++} tested.

Characterization of EAC^h and EAC^m

The rosette formation of C3 receptor bearing cells is utilized as one of the cell markers. To do this the usage of purified complement components is most advisable, but it is too difficult to do so in every laboratory. In general are used EAC^h or EAC^m made from human or mouse sera as complement source. And we tried to characterize these indicator cells in respect of the reactivity with various

Fig. 3. The reactivity of EAC^h and EAC^m with various cells. a, B lymphocytes; b, monocytes; c, PMN; d, human erythrocytes; $\square$ in the presence of EDTA; $\blacksquare$ in the presence of cations.

Fig. 4. The reactivity of trypsin-treated EAC^m with various cells. a, Daudi cells; b, B lymphocytes; c, monocytes; d, PMN; $\circ$, $\bullet$, presence or absence of 40 mM EDTA respectively.

cells and its dependence on divalent cations. As shown in Fig. 3, EAC^h reacted with human erythrocytes, B lymphocytes, PMN and monocytes. EAC^h prepared as described in this paper seems to react selectively with C3bR. The reaction of EAC^h with cells occurred independently of the existence of cations. EAC^m formed rosettes with B lymphocytes, PMN and monocytes, but those with the latter two were inhibited by EDTA. These results are quite identical with those obtained by EACI-3bi . EAC^m ($1 \times 10^8/\text{ml}$) were further treated with an equal volume of trypsin at 37°C for 30 min, followed by treatment with SBTI. The treated EAC^m were examined for their reactivity with various cells in the presence or absence of EDTA (Fig. 4). Higher concentrations of trypsin abolished the reactivity of the treated EAC^m with monocytes or PMN which does not express C3dR, while they maintained the reactivity with Daudi cells or B lymphocytes. EAC^m treated with lower concentrations of trypsin reacted with monocytes or PMN only in the presence of divalent cations. These results suggest that EAC^m prepared as described in this report has the similar reactivity to that of EACI-3bi^h , and by the treatment with trypsin it is converted to the indicator with reactivity similar to EACI-3d .

DISCUSSION

Some discrepancies concerning the reactivities of white blood cells with C3 bearing indicators are caused by the diversity of the state of bound C3 on indicator cells and the reaction conditions employed in rosette formation, especially in respect of the presence of divalent cations. We investigated the rosette formation of various human blood cells with EACI-3b , EACI-3bH , EACI-3bi and EACI-3d made from purified complement components, and their dependency on divalent cations.

The data presented in this paper show that B lymphocytes react with EACI-3b , EACI-3bH , EACI-3bi and EACI-3d , and these rosette formations do not require any divalent cations. In contrast, the rosette formation of PMN and monocytes with EACI-3bi requires Mg^{++} , although they do not require cations in the reaction with EACI-3b and EACI-3bH . Calcium ion gives synergistic effect with Mg^{++} on their reaction with EACI-3bi , but Ca^{++} gives no effect by itself. EACI-3d reacted with B lymphocytes and lymphoblastoid cell lines, Raji and Daudi, but not with PMN or monocytes irrespective of the presence of Mg^{++} . The fact that lymphocytes do not require Mg^{++} in contrast to PMN and monocytes may suggest that Mg^{++} may play a role in the expression of receptor for C3bi on these cells rather than to affect directly on the receptor-ligand reaction. The preliminary data that pretreatment of these cells with EDTA abolished the reactivity with EACI-3bi may support this idea. We have no evidence how Mg^{++} helps to express C3bi receptors on PMN and monocytes. However, the following possibilities might be considered. 1) Mg^{++} leads to unfolding of the cell membrane to express receptors that would have otherwise remained masked. 2) Mg^{++} causes

association or aggregation of receptors with consequent enhancing of the reactivity with EACI-3bi. 3) Mg^{++} regulates the release of elastase which cleaves C3bi to C3d (Ross and Lambris 1982). Phagocytes can move to the sites of inflammation, and chemotactic agents or anaphylactic reaction products such as ECF-A tetrapeptides or histamine are known to enhance the expression of C3 receptors (Anwar and Kay 1978). The fact that the expression of C3 receptors of phagocytes are controlled by various biological products would imply that these C3 receptors should have important roles in their cell functions. There are many cases where Mg^{++} is needed in cell-cell interaction. For example, lysis of antibody-dependent tumor cells by mouse macrophages is dependent on Mg^{++} (Esumi-Kurisu et al. 1980). In some of these cases it might be possible that C3 receptors play a role in their cell function.

We found that Raji cells did not react with EACI-3b but did with EACI-3bH (Okuda and Tachibana 1980). Lambris et al. (1980) observed the same phenomenon as we reported, and showed evidence that Raji cells have receptor for factor H and release endogenously synthesized factor I responding to the binding of factor H to its receptors. Thus the released factor I cleaves bound C3b into C3bi, and formed rosettes with Raji cells through C3bi receptor or C3d receptor. But we cannot think that the conversion of C3bH to C3bi is essential for the adherence of EACI-3bH to Raji cells to occur. Although the direct evidence are not yet available, the data presented in this paper will support the idea. EACI-3bH is different from EACI-3bi, because the former reacts with neutrophils or monocytes independent of cations but the latter reacts with them only in the presence of divalent cations. In addition, the presence of K76COONa and sodium azide did not affect the rosette forming activity of EACI-3bH with Raji cells (data not shown). K76COONa is known to block the cleavage of C3b to C3bi by I (Hong et al. 1979). Sodium azide blocks the release of I from Raji cells responding to the binding of H (Lambris et al. 1980). SDS-PAGE analysis showed that EACI-3bH recovered from the rosettes with Raji cells was not cleaved to EACI-3bi (data not shown).

Indicator cells, EAC^h and EAC^m , made from fresh human and mouse sera as complement source respectively are usually used to detect C3 receptors. We further characterized the state of bound C3 fragments of these indicators. EAC^h has a similar reactivity to that of EACI-3b^h. EAC^m reacts with B lymphocytes, Raji cells, but not with human erythrocytes. It reacts with PMN and monocytes only in the presence of Mg^{++} and by the treatment with trypsin it loses the reactivity with PMN and monocytes while preserving the reactivity with B cells and Raji cells. These results show that EAC^m is analogous with EACI-3bi^h and by the treatment with trypsin it becomes similar to EACI-3d^h.

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