COMPLEMENT BRIDGES BETWEEN CELLS

Analysis of a Possible Cell-Cell Interaction Mechanism*

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Cell-cell interaction is a basic topic in biological sciences. In embryogenesis, a largely unsolved problem is the means by which cell-cell recognition, necessary for the build up of a normal tissue, is mediated (1). Under pathological conditions this hitherto unrevealed mechanism of physiological interactions seems to be lost. In immunology numerous phenomena are based on cell-cell interactions. Thus, it is, for instance, well accepted that cell cooperation is required for the induction of an antibody response in B cells (2), as well as for the sensitization of T cells (3). In addition, the action of activated T cells on target cells is obviously a cell-cell interaction phenomenon (4). Macrophages are an essential participant in some of these interactions (5).

In this paper we want to outline in detail some mechanisms by which complement components might play a role in these cell-cell interaction phenomena. It will be demonstrated that complement factors located on one cell can be induced by enzymes on adjacent cells to form a bridge between these two cells.

Materials and Methods

Chemicals and Reagents. Trypsin, soybean trypsin inhibitor (SBTI),[1] and tannic acid (Merck A. G., Darmstadt, W. Germany). Except for C1 (guinea pig serum) the complement components used were from human serum. The components C1, C4, and C2 were purified to functional purity. C3 and C5 were purified to chemical purity by the method described by Tack and Prahl (6) applying polyethylene glycol during the initial step of the purification procedure. For some experiments we used labeled C3, the third component of the complement system with ^{125}I ($C3^x$) (7). C3b was generated from C3 or $C3^x$ according to Bokisch et al. (8). After completion of the experiments reported in this paper, for comparison, C3 was prepared by a method that avoided the use of polyethylene glycol (see Discussion).

Buffers. Isotonic veronal-buffered saline (VBS) pH 7.4, containing 1.5×10^{-4} M Ca^{++} and 1×10^{-3} M Mg^{++} was used as a general diluent for complement components and the sensitized and

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[1] *Abbreviations used in this paper*: C1, C2, C3, C4, and C5, first five components of complement; C3RC, C3 receptor-carrying cell; $C3^x$, third component of complement labeled with ^{125}I; $C\overline{42}$, C3 convertase; $C\bar{1}$, activated C1; EA, sensitized sheep erythrocytes; EAC1, EAC14, EAC142, EAC1423b, and EAC43b, EA coated with human complement components; $EAC43d^{hu}$, $EAC43b^{hu}$ treated with a C3b inactivator reagent; E^{tan}, tannic acid-treated sheep erythrocytes; E^{tan}-trypsin, trypsin-charged E^{tan}; LBS, labile binding site on nascent C3b; PBL, peripheral blood lymphocytes; SBS1 or SBS2, stable binding site one or two on the C3 molecule; SBTI, soybean trypsin inhibitor; VBS, veronal-buffered saline.

complement-coated erythrocytes. Eagle's MEM pH 7.2 (Autopow Flow Laboratories, Bonn) was used as a general diluent for lymphocytes.

Cells. Cells from three different human cell lines, Raji, Daudi, and Rael (culture seeds were kindly supplied by Dr. G. Klein, Stockholm), all derived from Burkitt lymphoma, were grown in RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with glutamine, nonessential amino acids, pyruvate, 10% fetal calf serum, penicillin; streptomycin, and Fungizone (9). Cells taken from mid-log phase cultures (5–8 $\times$ 10^5 cells/ml) were washed once with MEM, resuspended in MEM, and adjusted to a concentration of 2 $\times$ 10^6 cells/ml. Cell viability was assessed by the uptake of trypan blue.

Human peripheral blood lymphocytes from normal individuals were prepared from heparinized blood (10). Granulocytes were obtained from guinea pigs after intraperitoneal glycogen stimulation. Sensitized sheep erythrocytes (EA), EAC1, EAC14, EAC142, EAC1423b, and EAC43b were prepared from sheep erythrocytes, rabbit anti-sheep hemolysin (Behring-Werke, AG, Marburg/Lahn, W. Germany), and purified complement components (11). For the preparation of C2a-containing intermediates in all cases oxidized C2 was used to generate $C\overline{42}$ enzymes with a much greater stability (12); the half-life time of the intermediates used was about 30 min. Nonoxidized C2 was used only to prepare EAC43b. EAC43d were obtained from EAC43b by treatment with a C3b inactivator reagent (13). Before use, the cells were washed three times in VBS and adjusted to a concentration of 1.3 $\times$ 10^8 cells/ml VBS.

Trypsin-charged sheep erythrocytes were obtained by incubating equal volumes of a 2.5% erythrocyte (E)-suspension in VBS and a 5% tannic acid solution (in 0.9% sodium chloride) for 10 min at 37°C. After washing (700 *g*, 10 min) and resuspending, the tannic acid-treated sheep erythrocytes (E^{tan}) were mixed with equal vol of solutions of 5–50 mg trypsin/ml VBS, rotated for 12 h at 4°C, washed three times with VBS, and adjusted to a concentration of 1.3 $\times$ 10^8 cells/ml in VBS. For control, trypsin solutions were replaced by buffer.

Rosette Formation. The reaction mixture, consisting of 200 μl Raji cells (2 $\times$ 10^6/ml MEM) + 200 μl EAC1423b (1.3 $\times$ 10^8/ml VBS), was put into 1-ml plastic beakers (Eppendorf, Hamburg) and rotated on a submersion rotator for 20 min at 37°C. The rosette formation tests with other lymphocytes and intermediates were performed as described for Raji cells and EAC1423b.

Bridge Formation Assay. The test consisted of two steps: as a first step 200 μl of a suspension of Raji cells or other leukocytes (= step I cells) (2 $\times$ 10^6/ml) were preincubated with a solution of a complement factor (C3, C5, C4, or C1) or buffer in Eppendorf tubes for varying time periods at varying temperatures (see Results). Then the cells were washed four times in MEM (10 min, 400 *g*) and finally resuspended in 200 μl MEM.

As a second step, these 200 μl of pretreated cells were mixed with 200 μl of a suspension of either EAC142, other intermediates, or trypsin-charged E^{tan} (E^{tan}-trypsin) (step II cells) (1.3 $\times$ 10^8/ml). After rotating this mixture for up to 20 min at 37°C on a submersion rotator, cell-cell interaction was determined by enumerating rosettes which had been formed between one step I cell and three or more step II cells. The standard deviations were usually less than 10%.

Results

Formation of Complement Bridges between Cells by the Exclusive Use of Factors of the Complement System

C3 BRIDGES: BINDING OF EAC142 TO C3 RECEPTOR CELLS (C3RC), CARRYING UNCLEAVED C3. EAC1423b and EAC1423d can be attached to cells carrying receptors for the stable binding site (SBS1) on C3b or for the stable binding site (SBS2) on C3d (13, 14). In this case, C3 is used in its fragmented form, C3b or C3d, with both fragments bound to EAC142 through the labile binding site (LBS), which is transiently present on freshly generated C3b (15). In contrast to this standard procedure, C3-dependent interlinkage of cells can be induced by reversing the sequence of events, as shall be demonstrated in the following experiments. 200 μl of a suspension of lymphocytes, human PBL as well as Rael, Raji, and Daudi lymphoblastoid cells, or granulocytes^{gp}, respectively,

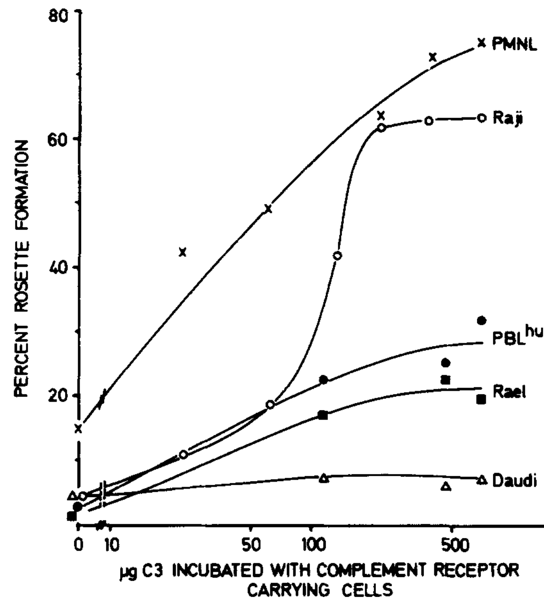

FIG. 1. Binding of EAC142 to leukocytes, carrying uncleaved C3. 0.4×10^6 leukocytes in 200 μl MEM were preincubated (20 min, 37°C) with increasing amounts of C3, washed four times, resuspended in 200 μl MEM, and tested for rosette formation with 200 μl of a suspension of EAC142 (1.3×10^8/ml).

were preincubated for 20 min at 37°C with 10–300 μl of a solution of uncleaved C3 (2.5 mg/ml). After this incubation the cells were washed four times, resuspended in 200 μl MEM, and mixed with 200 μl of a suspension of EAC142 (1.3×10^8/ml; carrying on an average 1.5 hemolytic sites of C2a per EAC142). This mixture was incubated in a submersion rotator for 20 min at 37°C. For the Raji cells the experiments were performed with C3, which had been labeled with ^{125}I to determine the amount of C3 molecules bound per cell. Fig. 1 shows the results.

Incubation of the different leukocytes with increasing concentrations of uncleaved C3 led to interlinkage of the C3RC and EAC142 as revealed by an increasing percentage of rosette formation between the C3RC and EAC142. Raji cells reached a saturation point at high C3 concentrations (2×10^7 C3 molecules/cell resulting from incubation of the Raji cells with 200 μg C3). More C3 did not lead to increased binding of EAC142. Within the C3 concentrations which were applied, guinea pig polymorphonuclear leukocytes, human PBL, and Rael cells also appeared to reach their maximal reactivity. Daudi cells behaved differently from the other cells. Even after incubation in solutions with a high C3 concentration they reacted weakly.

The control experiments, i.e. preincubation of the C3RC in medium instead of C3, resulted in less than 5% rosette formation except for the granulocytes of which 15% rosetted directly with EAC142. To rule out the possibility that the interaction between Raji-C3 cells and EAC142 was due to C3, which detached from the Raji cells, reacted with EAC142, and only then mediated classical C3-dependent rosettes, the following experiments were performed. 0.5 ml of a

suspension of Raji cells (2×10^6/ml) was preincubated (20 min, 37°C) with 0.5 ml of a C3 solution (2.5 mg/ml), washed four times, resuspended in 0.5 ml MEM, and incubated for additional 20 min at 37°C. 200 μl of the supernate of this incubation mixture was offered to 200 μl of a fresh Raji cell suspension. These cells were processed as above, but no C3-dependent interlinkage between Raji cells and EAC142 was observed. This fitted well with the fact that Raji cells which had been loaded with ^{125}I-C3^x (20 min, 37°C), washed four times, and incubated again at 37°C for 20 min hardly released any C3^x during this secondary incubation.

TIME AND TEMPERATURE DEPENDENCE OF THE C3 BINDING TO C3RC: The C3-induced interlinkage between C3RC and EAC142 was not only dependent on the concentration of C3 but also on the time and temperature of the period during which the C3RC were exposed to C3. This was demonstrated by incubating 200 μl of a Raji cell suspension (2×10^6/ml) with 100 μl of a C3 solution (2.5 mg/ml) for 1–40 min at 37°C and for 20 min at 4°C up to 37°C. Then the cells were washed four times and reacted with EAC142 as described in the previous chapter (Fig. 2). After 20 min at 37°C the maximal C3 effect on Raji cells was reached, which in a secondary incubation allowed optimal attachment of EAC142 (70% rosetted cells). Prolonging the preincubation by 20 min was disadvantageous, i.e. it resulted in 20% less rosette formation. The temperature effect was clearly demonstrable, in that increasing temperatures led to increasing C3 effects, namely from 10% rosettes after preincubation at 4°C to about 80% rosettes after incubation at 37°C.

ESSENTIAL ROLE OF UNCLEAVED C3 FOR THE C3-DEPENDENT BRIDGE FORMATION. The experiments performed thus far were compatible with the assumption that the two stable binding sites of C3 (SBS1 and SBS2) as well as the LBS are involved in the C3-dependent bridging reaction. To check directly for the relevance of the LBS we applied C3b, which had been generated in the fluid phase and thus had been deprived of LBS. 200 μl of a Raji cell suspension was incubated (20 min, 37°C) with increasing amounts of radiolabeled C3b^x or C3^x, respectively. After the cells had been washed, they were reacted with EAC142 and in parallel experiments with EAC1423b (Fig. 3).

As demonstrated in Fig. 3, C3b was unable to modulate the lymphoid cells so that they would bind EAC142 effectively. Nevertheless C3b was capable of occupying the C3 receptors on Raji cells so that they did not form rosettes with EAC1423b anymore. In contrast, while also blocking the C3 receptors, C3 was able to induce binding of EAC142 (see also Figs. 1 and 2), stressing the importance of uncleaved C3 for the binding reaction.

C5 BRIDGES: BINDING OF EAC142, EAC1423B, AND EAC43B/D TO C3RC-CARRYING C5. The discovery of the C3-dependent bridges stimulated the interest in searching for a role of other complement components. This paragraph deals with C5. In the same manner as for C3, the Raji cells were incubated with a C5 solution (5 mg/ml) and then tested with different intermediates (Fig. 4). Great care was taken to assure that C5 was free of C3. Thus, even if the C5 pools used were highly concentrated no precipitation with anti-C3 serum could be observed. At the same time in hemolytic assays no C3 could be found.

As depicted in Fig. 4: EAC142 bound to Raji cells best with increasing amounts of C5 offered during the preincubation period. Without modulation by

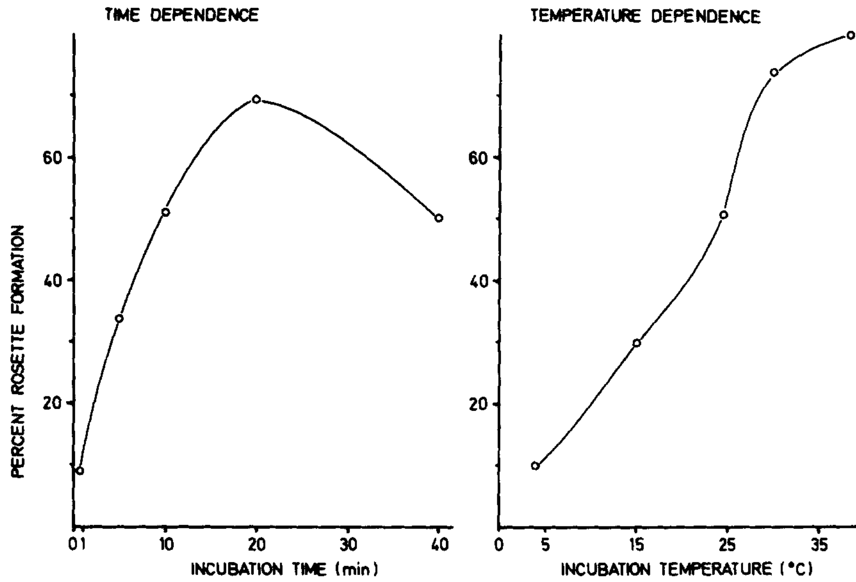

FIG. 2. Time- and temperature-dependence of C3-induced bridging between Raji cells and EAC142. 0.4 × 10^6 Raji cells in 200 μl MEM were incubated for 1-40 min at 37°C and for 20 min at 4°-37°C with 100 μl of a C3 solution (2.5 mg/ml), washed four times and tested for rosette formation with EAC142.

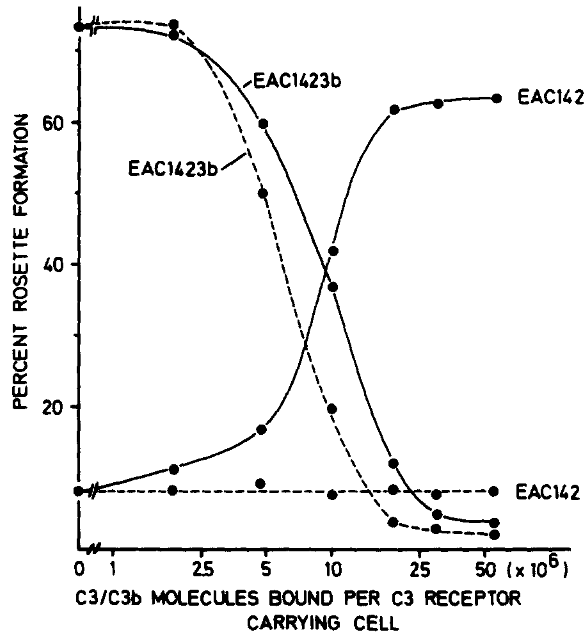

FIG. 3. Essential role of uncleaved C3 for the C3-dependent bridge formation. Samples of 0.4 × 10^6 Raji cells in 200 μl MEM were incubated (20 min, 37°C) with radiolabeled $C3^x$ (——) or $C3b^x$ (-----), respectively, washed four times and resuspended in 200 μl MEM. The Raji cells, carrying different amounts of C3 or C3b molecules per cell, were tested for rosette formation with EAC142. For controls, rosette formation tests with EAC1423b were included.

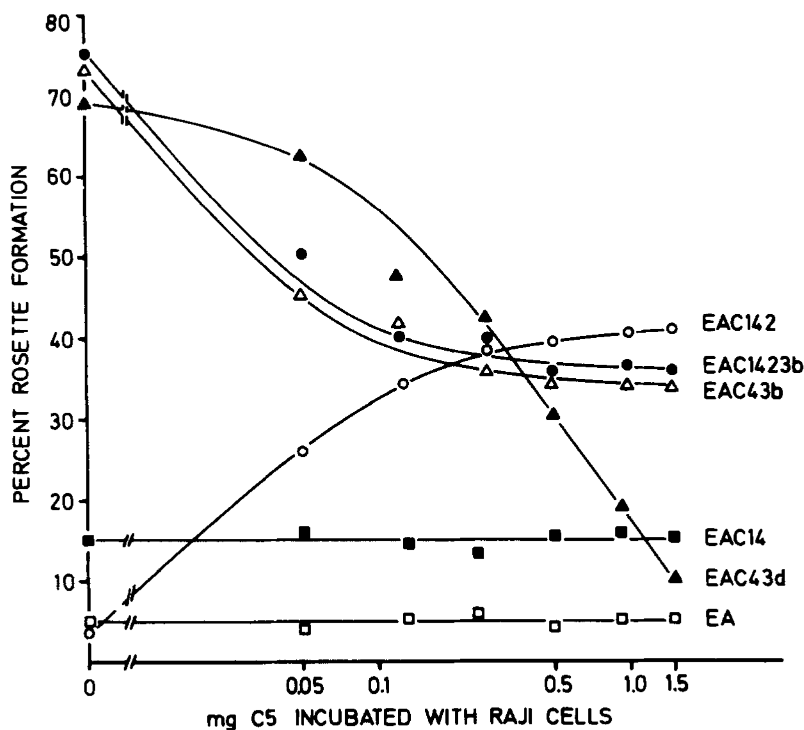

FIG. 4. C5-induced interlinkage between Raji cells and different EAC intermediates. Samples of 0.4×10^6 Raji cells in 200 μl MEM were incubated with increasing amounts of C5 for 20 min at 37°C, washed four times, resuspended in 200 μl MEM and tested for rosette formation with various EAC intermediates.

C5 no attachment occurred. In contrast to this result, EAC1423b, EAC43b, and EAC43d behaved differently. They bound, as expected, to Raji cells which had not been exposed to C5. With all three intermediates this binding was decreased by preincubation of Raji cells with C5. In the case of EAC1423b and EAC43b, formation of about 70% rosettes was lowered to about 35%, whereas in the case of EAC43d rosette formation was reduced even further. EA and EAC14 did not support C5-dependent cell-cell interlinkage.

DEPENDENCE OF C3- AND C5-BRIDGES ON THE CONCENTRATION OF $C\overline{42}$ ENZYMES PER EAC142. In the preceeding chapters it was demonstrated that bridge formation between C3- or C5-coated Raji cells and EAC142 clearly depended on the concentration of C3 (Figs. 1 and 3) or C5 (Fig. 4). The following experiments shall clarify the importance of the concentration of $C\overline{42}$ sites per EAC142. Raji cells were incubated (37°C, 20 min) with an optimal amount of C3 (corresponding to 4×10^5 Raji cells + 250 μg C3 in a vol of 300 μl) or C5 (4×10^5 Raji cells + 500 μg C5 in 300 μl), washed four times, and resuspended to 2×10^6 cells/ml. Samples of these suspensions were then tested in the bridge formation assay with EAC14, which had been prepared with various concentrations of oxidized C2.

The results show (Fig. 5) that the concentration of $C\overline{42}$ enzymes per EAC142 determined the degree of bridge formation with C3- or C5-coated Raji cells.

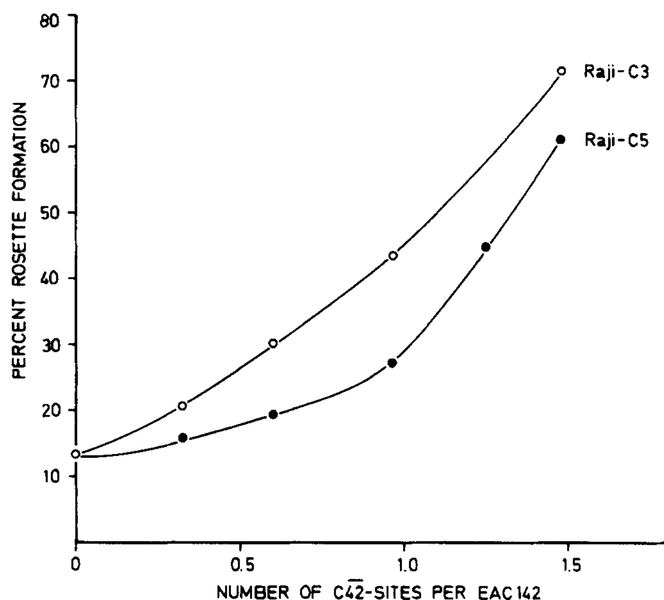

FIG. 5. Dependence of the bridge formation between C3- or C5-coated Raji cells and EAC142 on the amount of C$\overline{42}$ enzymes per EAC142. Samples 0.4×10^6 C3- (○——○) or C5- (●——●) coated Raji cells (in 200 μl MEM) were mixed with 200 μl of EAC14 (1.3×10^8/ml), to which various amounts of C2 had been attached (abscissa). After 20 min at 37°C the percentage of rosette formation (ordinate) was enumerated.

EAC142 with low numbers of hemolytically active C$\overline{42}$ enzymes seemed to cooperate more effectively with C3-coated Raji cells than with cells coated with C5. At higher C$\overline{42}$ concentrations this was less obvious.

C4 BRIDGES: BINDING OF EAC1 TO C3RC-CARRYING C4. To investigate the potential role of C4, Raji cells were preincubated with a C4 solution (5 mg/ml) and then tested with a suspension of EA, EAC1 (carrying about 300 C1 molecules per cell), EAC14, EAC142, and EAC1423b (1.3×10^8/ml). The results are described in Fig. 6. For controls Raji cells were preincubated in buffer instead of C4.

In the absence of C4, Raji cells formed rosettes with EAC1423b (70%), EAC1 (30%), and EAC14 (15%), whereas the interaction with EAC142 (5–7%) and EA (5%) was very weak. Preincubation of Raji cells with C4 led to differing patterns for the different intermediates. Increasing C4 concentrations resulted in decreasing percentages of rosettes between Raji cells and EAC1423b while the interaction of Raji cells with EAC1, EAC14, and EAC142 was improved in a dose-dependent fashion. EAC142 responded at lower C4 concentrations on the Raji cells with an increase of rosette formation as did EAC14 or especially EAC1, the latter needing about 10 times as much C4 during the preincubation to show a noticeable reaction. EA remained unaffected even at high C4 concentrations.

C1 BRIDGES: BINDING OF EA TO CELLS CARRYING C1. The data depicted in Fig. 6 shows that Raji cells did not interact with EA but with EAC1 and EAC14. Here the reaction sequence is reversed and Raji cells are coated first

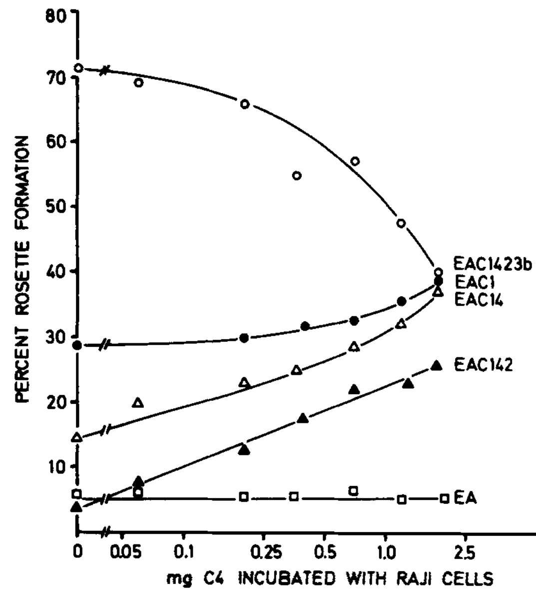

FIG. 6. C4-induced interlinkage between Raji cells and different EAC intermediates. Samples of 0.4×10^6 Raji cells in 200 μl MEM were incubated with increasing amounts of C4 for 20 min at 37°C, washed four times, resuspended in 200 μl MEM, and tested for rosette formation with various EAC intermediates.

TABLE I

C1-Induced Bridge Formation between Raji Cells and EA

μg C1 incubated with Raji cells	Rosette formation
	%
0	5
18	18
36	22
54	27

with C1 and then interacted with erythrocytes. The dependence of the bridge formation on the dose of C1 is described.

200 μl of a Raji cell suspension (2×10^6/ml) was incubated with 100–300 μl of a C1 solution (360 μg/ml). After 15 min at 37°C the cells were washed four times, resuspended in 200 μl MEM, and run in the rosette assay with EA (Table 1). Table 1 shows that C1, in a dose-dependent fashion mediated interaction of EA with Raji cells. If EA was replaced by E, a reaction was not observed.

Induction of Complement Bridges by the Use of an Unspecific Protease. In the experiments outlined in the preceeding paragraphs, cell-cell interactions were described, which were based on the cooperation between uncleaved complement components (C3, C5, and C4) and enzymes of the complement system, such as $\overline{C42}$ and $C\overline{1}$. It is assumed that activation of the uncleaved complement factors was essential for the reaction. Since in fluid phase systems

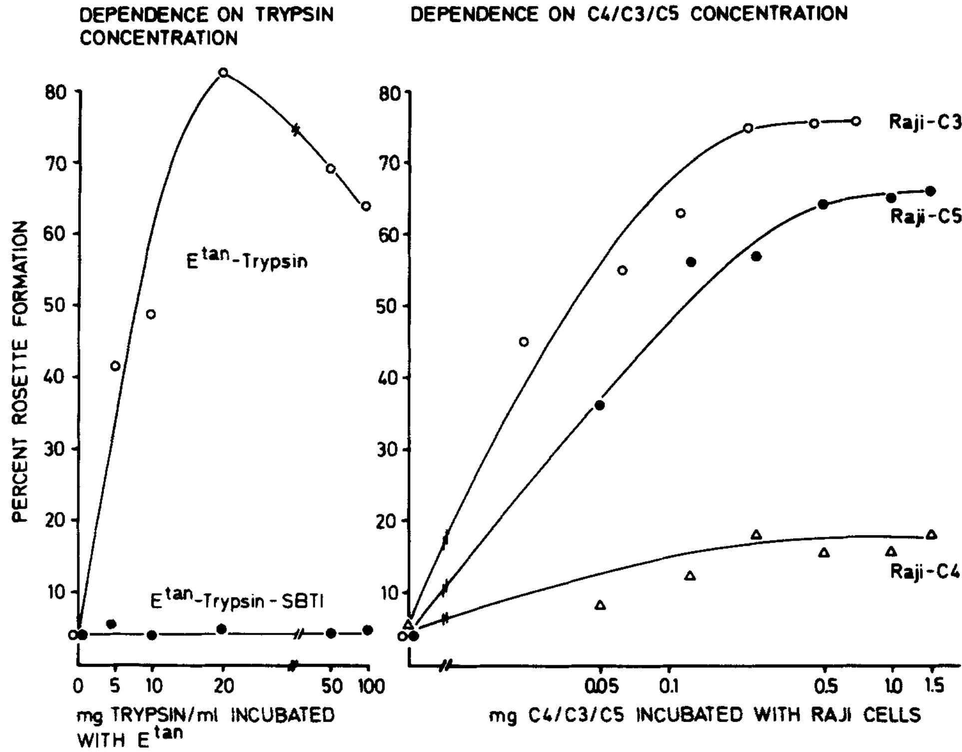

FIG. 7. Bridge formation between Raji cells coated with complement factors and E^{tan} carrying trypsin. Left part: Raji cells coated with C3 were tested for rosette formation with E^{tan} carrying different amounts of trypsin. For control E^{tan}-trypsin were treated with SBTI. Right part: Samples of 0.4×10^6 Raji cells in 200 μl MEM were incubated (20 min, 37°C) with increasing amounts of C3, C5, or C4, respectively, washed four times, resuspended in 200 μl MEM and tested for rosette formation with E^{tan}-trypsin (prepared with a trypsin solution of 20 mg/ml).

unspecific proteases have been used to activate complement components (8, 16, 17), we investigated the possibility that complement-dependent bridge formation could also be induced by an unspecific protease like trypsin. Therefore 200 μl of a Raji cell suspension (2×10^6/ml) was incubated with an optimal amount of C3 (100 μl of a C3 solution of 5 mg/ml) for 20 min at 37°C. When the cells had been washed four times, they were resuspended in 200 μl MEM and incubated in the rosette assay with 200 μl of a suspension of E^{tan}-trypsin (1.3×10^8/ml). The E^{tan}-trypsin had been prepared by using different concentrations of trypsin. For control, trypsin inhibitor solutions were added to the reaction mixture (Fig. 7).

As demonstrated in Fig. 7, increasing trypsin concentrations during the preparation of E^{tan}-trypsin led to higher activity in the bridge formation assay. But concentrations above 20 mg trypsin/ml resulted in E^{tan}-trypsin, which showed a lower reactivity. In all instances the trypsin inhibitor, the concentration of which in each case marked the original trypsin concentration, was able to block the reaction completely. To demonstrate that this reaction was clearly dependent on C3 and could also be induced with other complement components

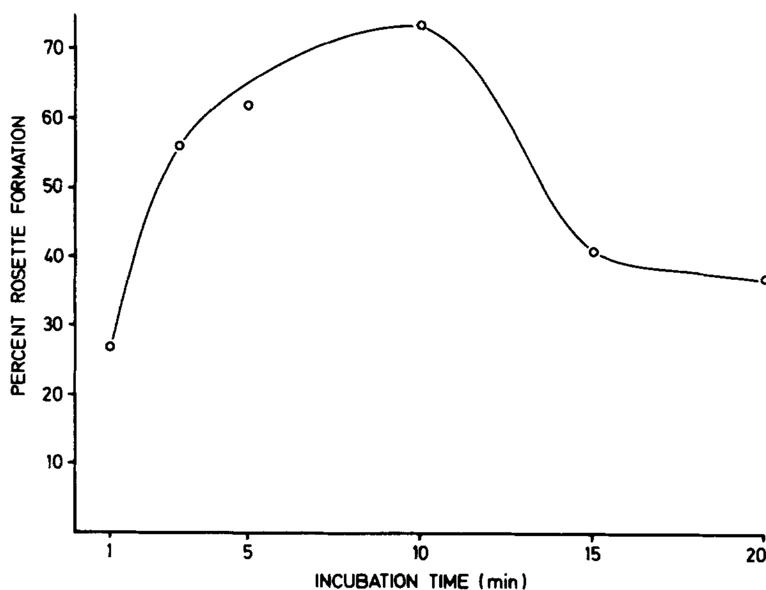

FIG. 8. Time-dependence of the interlinkage between C3-coated Raji cells and E^{tan}-trypsin. Raji cells coated with C3 were incubated for 1–20 min at 37°C with E^{tan}-trypsin (prepared with a trypsin solution of 20 mg/ml) to determine the rosette formation profile.

such as C5 or C4, samples of Raji cells were incubated with C3, C5, or C4, respectively. The different sets of cells were washed four times and every cell sample was resuspended in 200 μl MEM. These cell samples were then mixed with 200 μl of E^{tan}-trypsin (1.3×10^8), which had been prepared with 20 mg trypsin/ml.

After rotation for 10 min at 37°C rosettes were enumerated (Fig. 7). As demonstrated in Fig. 6, dependent on the concentration of C3, C5, or C4, with which the Raji cells had been preincubated, rosettes were formed. C3 and C5 were about identical with respect to their capacity to support this interaction (65–75% rosettes). C4 reacted weaker and induced only up to 20% rosettes.

The interaction of E^{tan}-trypsin with Raji-C3 was time-dependent. Raji-C3, prepared as for the experiments depicted in Fig. 6, and E^{tan}-trypsin, prepared with 20 mg trypsin/ml, were incubated at 37°C for 1–20 min (Fig. 8). The reaction was optimal after 10 min and decreased clearly from there on.

Discussion

The data presented above show that complement components can form bridges between cells: once uncleaved complement factors like C3, C5, or C4 had been attached to cells carrying appropriate receptors, proteases on adjacent cells were able to activate these factors so that they acquired the capacity to anchor the complement factor-carrying cell to the protease-carrying cell. Thus, complement factors and proper proteases formed bridging units like: C3 + $C\overline{42}$, C5 + $C\overline{42}$, and C4 + $C\overline{1}$. In addition to these pure complement formations, units could be built from C3, C5, or C4 plus an unspecific protease like trypsin and from C1+ antibody. The analysis of this mechanism has to provide an

explanation for the binding of uncleaved complement factors to the surface of the different cells and for the protease-induced activation of these factors resulting in a complement bridge.

The binding of the uncleaved complement factors to cell surfaces is not yet completely understood. C3, C5, and C4 are attached at least in part to C3 receptors since, once they are bound to C3RC, the latter form less rosettes with EAC1423b (Figs. 3, 4, and 6) indicating impaired C3 receptor function. But besides the binding to C3 receptors, complement factors are possibly fixed to other sites, since Rael cells, which form no rosettes with EAC1423, are capable of binding C3 so that C3-dependent bridges can arise (Fig. 1). Irrespective of the site on the cell surface, it appears to be critical to bind C3 in the proper way. Daudi cells, having C3d receptors, can attach C3 via its stable binding site two, SBS2 (located on the C3d part) (14), but are unable to give rise to C3-dependent bridges (Fig. 1). Thus, it seems to be of major importance to bind C3 through its SBS1 (associated with the C3c part) (14) to C3b receptors, possibly to leave the C3a end freely accessible (see scheme below). It should be mentioned here that binding of uncleaved, purified C3 to C3RC is in contrast to the fact that uncleaved C3 in whole serum does not bind to C3RC. But we observed that during the C3 purification C3 gained this binding capacity (unpublished results). The fact that this occurred early during the C3 purification procedure argues against partial denaturation of C3 and favors separation of C3 from factors which cover it as an explanation for the binding. The same consideration may hold true for C4 and C5. The possibility that for the C3 reactions discussed here C3b might be responsible was ruled out, because it could be shown that C3b was able to bind to C3 receptors but unable to mediate bridge formation (Fig. 3). The amount of C3 molecules that could be attached to the C3RC (Fig. 3) was repeatedly found to be surprisingly high, especially in comparison to an earlier report (18), suggesting that binding of C3 to Raji cells was limited to about 5×10^5 molecules per cell. However, in detailed binding studies even with highly concentrated C3 solution, we were unable to reach a clear saturation point (unpublished data). Comparing the potency of isolated C3 to induce bridge formation (Raji-C3 + EAC142) or to inhibit rosettes between Raji cells and EAC1423 it was observed that those concentrations of C3 which allowed complete inhibition of rosette formation were also the concentrations allowing maximal bridge formation. In this context it is of interest that in the report cited above (18) amounts of C3 were needed for complete rosette inhibition which were similar to those used in our experiments for optimal bridge formation (Fig. 1). We would like to add, that C3 purified in different ways might behave differently. Thus, for the preparation of C3 used in the experiments reported here, we used polyethylene glycol, following the method of Tack and Prahl (6). After completion of the experiments reported here we prepared C3, starting with separation of euglobulins and pseudoglobulins instead of using polyethylene glycol. Such a C3 shows the same reactions with respect to bridge formation as well as rosette inhibition but only about $^1/_5$–$^1/_{10}$ of the amount of C3 is necessary for the same degree of response (unpublished results).

The binding of C3 to the C3RC so that C3 can support bridge formation was a

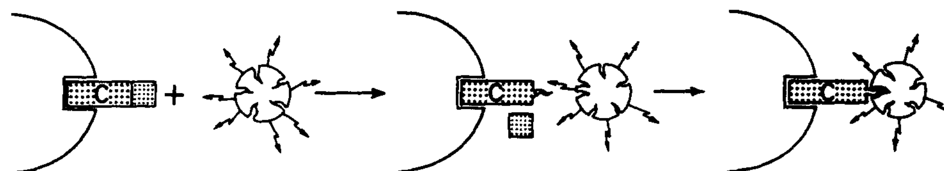

FIG. 9. Complement bridges between cells: scheme of the suggested mechanism. Surface proteases (↗) liberate LBS (➤) on complement factors (C), which mediate the bridge formation.

time- and temperature-dependent process (Fig. 2). Although C3 was bound to C3 receptors at 4°C (19), higher temperatures during the preincubation resulted in more bridges. In analogy to requirements of the rosette formation (19), it is reasonable to assume that the higher temperature was required to allow the lateral mobility of the C3 receptors, so that after binding of C3 they could gather in patches and eventually in caps. This may be advantageous only up to a certain point beyond which it could possibly result in a smaller number of bridges as demonstrated by fewer rosettes after prolonged incubations at 37°C (Fig. 2).

The activation of the surface-bound C3 to form bridges most likely occurs in the same manner as activation of C3 in the fluid phase: C3 on one cell is cleaved by the C$\overline{42}$ enzyme on an adjacent cell into C3a and C3b; the LBS on C3b is liberated and anchors the surface-bound C3b onto the adjacent cell, thus interlinking the two cells (see model in Fig. 9). It can be assumed that numerous C3 molecules have to participate to make it an effective reaction. Thus far we cannot produce direct evidence that proteolytic cleavage of the surface-bound C3 molecules is essential. But the simultaneous requirement of the complement components and of the proteolytic activity (Figs. 4–7) for the bridge formation to occur leaves no doubt. This mechanism is not restricted to units consisting of C3 + enzyme but functions also with other complement factors. Thus, surface-bound C5 can be activated by the C$\overline{42}$ enzyme to interlink the C5 carrier with the carrier of C$\overline{42}$, so that the unit consists of C5 + C$\overline{42}$ (Figs. 4 and 5). As the underlying mechanism we propose cleavage of C5 into C5a and C5b, the latter remaining attached to the cell but exposing an LBS. This LBS, as in the case of nascent C3b, would then anchor the C5b-carrying cell to the protease-carrying cell (see model in Fig. 9). The cleavage of C5 by activated C2 is well established for the classical activation sequence as it occurs in erythrocyte lysis (20), although in the classical pathway C3b (EAC1423) is needed probably to locate C5 close to the membrane and possibly to alter C5 so that it can be cleaved by C2. At first glance, these requirements seem not to be fulfilled under the special conditions of the bridge formation experiments. But it is not at all clear that binding of C5 to the surface of the lymphoblastoid cells might not possibly result in such an alteration of C5 so that it now can be cleaved by C2. It has also to be kept in mind that the EAC142 used in our experiments could possibly carry a small number of C3b molecules

which cannot be detected hemolytically but which could serve as initial attachment sites for the membrane-bound C5. The binding of C5 to the lymphoid cells in all likelihood involves the C3 receptors, since after incubation of C3RC with C5 the C3RC form less rosettes with EAC1423b (Fig. 4). In addition, E-C5 form rosettes with Raji cells and these rosettes can be inhibited by C3 (unpublished data; a detailed account of the C5 binding modalities will appear separately). If one accepts C5 binding to C3 receptors then one would expect that C3-dependent rosette formation would be blocked completely by high C5 concentrations. But blocking of C3b-dependent rosette formation is possible only to a certain degree and then reaches a plateau (EAC1423b and EAC43b in Fig. 4). One explanation might be that uncleaved C5 while occupying the C3 receptors is capable of binding to C3b. The bridging unit thus would consist of C5 + C3b. This is supported by the fact that EAC43d do not interact with Raji-C5 (Fig. 4).

With respect to C4 a proper bridging unit appears to consist of C4 + $C\bar{1}$ (Fig. 6). In the course of these experiments some interesting features were revealed. EA gain reactivity with Raji cells when they are transformed to EAC1, a reaction which clearly depends on the amount of C1 molecules (unpublished data) (25–30% rosettes were formed in case of 300 C1 molecules per EA); EAC14 show 15% and EAC142 only about 3% rosette formation. The reactive site of EAC1 mediating binding to Raji cells is either lost during the incubation needed to prepare the higher intermediates or is covered by C4 and C2. The interaction between Raji cells and EAC1 could be due to the receptor for C1q, which has been described for lymphoid cells (21). At the same time it is tempting to attribute a role to the C4, found in Raji cell membranes (22). It is not understood why EAC1 need higher C4 concentrations to respond in the bridging assay than EAC14 and especially than EAC142. The question is raised if possibly C2a in the EAC142 plays a role by either cleaving C4 or offering a site for C4 attachment. The sites to which the uncleaved C4 molecules are bound on Raji cells seem to be at least in part the C3 receptors, since C4 binding is followed by reduced C3-dependent rosette formation (Fig. 6). Such an assumption is supported by the earlier report that C4b can interfere with C3 receptor function (23).

Based on the presence of C1q receptors on Raji cells (21) and the finding that EAC1 bound to Raji cells, it was not so surprising that Raji cells could be coated with soluble C1, so that C1 in a dose-dependent manner would induce rosettes between Raji-C1 and EA in the bridge formation assay. In this case the interacting unit was C1 + antibody.

The bridge formation is not initiated solely within the complement system. The data show that the $C\overline{42}$ enzyme can be replaced by an unspecific protease like trypsin. The effect is dependent on an optimal trypsin concentration (Fig. 7) and an optimal incubation time (Fig. 8). C3 and C5 are better reaction partners (65–75% rosettes) than C4, which yields only a limited amount of bridging units (15–20% rosette formation between Raji-C4 + E^{tan}-trypsin). In view of the fact that in the fluid phase C3 can be activated by trypsin (8, 16, 17), similar activation processes might underly the trypsin-induced C3-, C5-, and C4-bridges. The schematic drawing in Fig. 9 describes the mechanism discussed here.

This mechanism of complement-dependent bridges between cells may be of great biological significance. It has been demonstrated that C4 (22), C1 (24), and factor B (25) are expressed in membranes. Anti-C4 antibodies can inhibit the mixed lymphocyte reaction (22). It seems very conceivable that complement factors, which are naturally exposed in cell membranes may be activated by a proper protease on an adjacent cell leading to cell-cell contact. Besides the possibility of induction of cell-cell contact through this mechanism it may be even more likely that two cells, which are in contact already through recognition of certain antigens on one cell by certain receptors on the other, become tightly connected through complement bridges. Expression of proteases on cell surfaces is described under different circumstances. Thus, macrophages upon activation show proteolytic activity on their surfaces (26–28). An increase in protease activity is also expressed in virus-infected cells (29). Our preliminary experiments with herpes virus-infected cells indicate that proteases become expressed which are capable of functioning in complement-dependent cell-cell contact.[2]

The possibility that a molecule on one cell through alteration by enzymes on an adjacent cell acquires the capacity to interlink these two cells is also discussed for other systems. Reaggregation of the cells of disrupted retinal tissues, for instance, may be related to such events (1; for additional references see 30). An important difference between such processes as reaggregation of cells of a specific tissue and complement bridges is that the complement bridges lack specificity, because the labile groups of C3 or C5, for instance, bind to every cell surface thus far investigated.

Nevertheless this process might have specificity in as much as the complement factors might be available only on certain cells and/or the activating proteases could contribute to specificity by limited representation on cells and restricted enzymatic activity.

Summary

Different leukocytes (Raji, Daudi, Rael lymphoid cells; human peripheral blood lymphocytes, and guinea pig granulocytes), which had been coated with C3 by incubation at 37°C for 20 min in a C3 solution, were demonstrated to form rosettes with erythrocytes coated with complement components (EAC142). The percentage of rosettes was dependent on the amount of C3 present on the cells. Loading of the lymphoid cells with C3 was a time- and temperature-dependent process. C3b was unable to serve the same purpose, although C3 and C3b occupied the C3 receptors on the lymphoid cells to a comparable degree. C5 functions in a similar manner. The C$\overline{42}$ enzyme can be replaced by trypsin, so that bridging units may consist of C3 + C$\overline{42}$, C5 + C$\overline{42}$ or C3 + trypsin, and C5 + trypsin. Bridging units can be constructed also from C4 + C1. It is suggested that enzymes on one cell liberate labile binding groups of complement components on adjacent cells, thus inducing coupling of the two cells. The possibility is raised that this type of cell interlinkage may play a role in vivo, since there is accumulating evidence that complement components are expressed in the plasma membrane of different cells.

[2] M. A. Dierich and B. Landen. Manuscript in preparation.

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