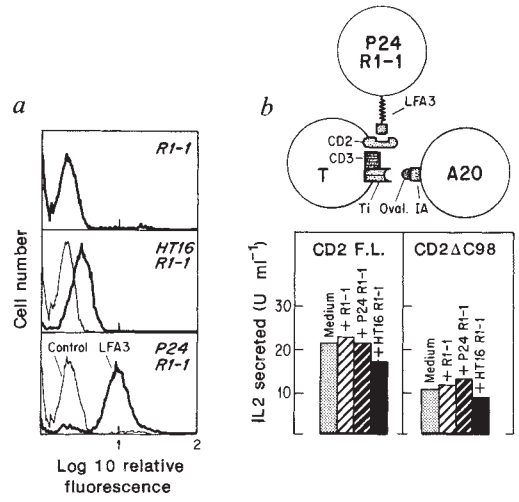

FIG. 3 Co-stimulation of CD2 transfected murine T cells with antigen-pulsed A20 cells plus LFA-3 expressing third party cells: requirement for LFA-3 expression on the cognate partner. *a*, Immunofluorescence analysis of human LFA-3 expression on transfected thymoma. P24R1-1 and HT16R1-1 were derived from the murine thymoma R1-1 by transfection with the full-length human LFA-3 cDNA encoding the phosphatidyl inositol-linked and transmembrane forms of the molecule, respectively, as described in Fig. 2. *b*, IL-2 production by $CD2^+$ transfectants upon antigenic stimulation in the presence or absence of third party cells.

METHODS. *a*, Cells were stained for 30 min at 4 °C with the anti-LFA-3 TS2/9 mAb (supernatant at a 1:5 final dilution), washed twice, and incubated with goat anti-mouse IgG second antibody (1:40 dilution, Meloy). Cells were analysed (10,000 per sample) on an Epics V cell sorter. Histograms represent reactivity with anti-LFA-3 MAb (thick line) compared with an equivalent amount of an irrelevant antibody (1HT4 4E5 ascites, 1:200 final dilution) (thin line). *b*, IL-2 assays were performed as in Fig. 2*c* but included, in addition, 50,000 R1-1 or HT16R1-1 or P24R1-1 cells per well. IL-2 production resulting from stimulation of 10^5 CD2 transfectants with 50,000 A20 cells plus ovalbumin alone are represented in the bar labelled 'medium'.

for CD2–LFA-3 interactions in cellular systems where the stimulus for the T cell is presented on a xenogeneic cell population (which fails to bind the endogenously expressed CD2 of the other species), or liposomes bearing purified alloantigen and LFA-3 molecules[8]. These latter systems have readily identified the synergistic effect of CD2–LFA-3 signal transduction on T-cell receptor triggered activation. In contrast to the CD2 adhesion function defined here, CD2-mediated signal transduction requires the CD2 cytoplasmic tail[8,12,13].

The augmented IL-2 production observed after stimulation of human $CD2^+$ murine T-cell clones by antigen and human LFA-3-expressing antigen-presenting cells probably results from the ability of the CD2–LFA-3 adhesion pair to facilitate more efficient formation of the ternary complex between the T-cell receptor and antigen plus the MHC gene product. Although the affinity of monomeric CD2–LFA-3 interactions is low (micromolar) the increased avidity resulting from multimeric interactions of multiple transmembrane CD2 copies on a given T cell with multiple LFA-3 molecules on the antigen-presenting cell is likely to be great and should serve to oppose, more optimally, surface membranes of the two cell types. Alternatively, if the interaction between LFA-3 and CD2 provides an as yet unrecognized signal that enhances IL-2 responsiveness independently of the known CD2 signalling mechanisms, then an LFA-3–CD2 interaction resulting from an unrelated third-party cell might enhance the functional consequences of T-cell receptor triggering, thereby resulting in enhanced IL-2 production. To test this possibility, cDNAs encoding the transmembrane form as well as the phosphatidyl inositol-linked form of LFA-3 were transfected into a thymoma cell line (R1-1), which does not express $I\text{-}A^d$, to yield cell lines termed HT16R1-1 and P24R1-1 respectively (ref. 17 and B.P.W., manuscript in preparation). As shown by immunofluorescence analysis (Fig. 3*a*), the phosphatidyl inositol-linked LFA-3 form is expressed at levels about fivefold higher than the transmembrane form. Each of these cell lines was tested for their co-stimulatory effect on human CD2 expressing murine T cells in conjunction with ova pulsed A20 (schematically depicted in Fig. 3*b*). A representative experiment is shown in Fig. 3*b* (bottom). Identical results are found at higher and lower third-party cell numbers (not shown). No significant differences were observed in IL-2 production following stimulation by ova +A20 cells, regardless of which third-party cell was added into the assay. Similar results were obtained using the CD2ΔC43 and 3DO54.8 IL-2 producing cells lines (data not shown). Thus, the adhesion mediated between CD2 and LFA-3 optimizes the physical interaction between the antigen-responsive T cell and its cognate partner.

Earlier studies using monoclonal antibodies directed against either the CD2 adhesion domain (anti-$T11_1$-like) or LFA-3 delineated a profound inhibition of helper and cytotoxic T-cell responses and thymocyte proliferative responses in *in vitro* assays[9,10,18]. The present results unambiguously define the importance of CD2–LFA-3 adhesion in such interactions even in the absence of CD2 signal transduction. Thus, the CD2–LFA-3 adhesion pair, like the CD4–MHC class II adhesion pair[19], augments T-cell response even in the absence of their respective CD2 or CD4 cytoplasmic segments. We presume that CD2 and LFA-3 function similarly to increase the interactions between a cytolytic T lymphocyte and its target cell. One can imagine that this adhesion is also important for thymocyte-thymic epithelial interactions involved in thymic education and differentiation. □

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1. Selvaraj, P. *et al. Nature* **326,** 400–403 (1987).
2. Shaw, S. *et al. Nature* **323,** 262–264 (1986).
3. Hunig, T. *et al. J. exp. Med.* **162,** 890–901 (1985).
4. Meuer, S. C. *et al. Cell* **36,** 897–906 (1984).
5. Brottier, J., Boumsell, L., Gelin, C. & Bernard, A. *J. Immun.* **135,** 1624–1631 (1985).
6. Hunig, T., Tiefenthaler, G., Meyer zum Buschenfelde, K. H. & Meuer, S. C. *Nature* **326,** 298–301 (1987).
7. Yang, S. Y., Chouaib, S. & Dupont, B. *J. Immun.* **137,** 1097–1100 (1986).
8. Bierer, B., Peterson, A., Gorga, J. C., Hermann, S. H. & Burakoff, S. J. *J. exp. Med.* **168,** 1145–1156 (1988).
9. Palacios, R. & Martinez-Maza, O. *J. Immun.* **129,** 2479–2485 (1982).
10. Tadmori, W. *et al. J. Immun.* **136,** 1155–1160 (1986).
11. Sanders, M. E. *et al. J. Immun.* **140,** 1401–1407 (1988).
12. He Q., Beyers, A. D., Barclay, A. N. & Williams, A. F. *Cell* **54,** 979–984 (1988).
13. Chang, H. C. *et al. J. exp. Med.* (in the press).
14. Sayre, P. H., Hussey, R. E., Chang, H. C., Ciardelli, T. L. & Reinhaerz, E. L. *J. exp. Med.* **169,** 995–1009 (1989).
15. Nagafuchi, A. & Takeichi, M. *EMBO J.* **7,** 3679–3684 (1988).
16. Yagita, H. *et al. Proc. natn. Acad. Sci. U.S.A.* **86,** 645–649 (1989).
17. Wallner, B. *et al. J. exp. Med.* **166,** 925–932 (1988).
18. Denning, S. M. *et al. J. Immun.* **139,** 2573–2578 (1987).
19. Sleckman, B. P. *et al. J. Immun.* **141,** 49–54 (1988).
20. Sayre, P. H. *et al. Proc. natn. Acad. Sci. U.S.A.* **84,** 2941–2945 (1987).
21. Haskins, K. *et al. J. exp. Med.* **157,** 1149–1169 (1983).
22. Johnson, G. D. & Holborow, E. J. in *Handbook of Experimental Immunology* Vol. IV (ed. Weird, D.) 1–21 (Oxford University Press, 1986).

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Rapid induction of neutrophil–endothelial adhesion by endothelial complement fixation

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THE adhesion of neutrophils to vascular endothelium is an early event in their recruitment into acute inflammatory lesions[1]. In evaluating potential neutrophil–endothelial adhesive mechanisms in acute inflammation, important considerations are that adhesion

in vivo **may occur very rapidly following injury[2–4] and that the specificity of the reaction resides in altered endothelium[5]. That is, neutrophils adhere only to altered endothelium adjacent to an inflammatory focus, rather than at random as would be expected if activation of neutrophils were the initiator of adhesion. We have explored a possible bridging role for complement in causing early neutrophil–endothelial cell adhesion. The complement system is involved in inflammatory processes, is capable of rapid amplification, and endothelial complement fixation at sites of inflammation could generate an endothelium-restricted signal for neutrophil adhesion. We have now developed a model in which this can be investigated without complicating factors such as immunoglobulin deposition, by constructing a novel molecule, a hybrid of the endothelial binding lectin *Ulex europaeus* I (ref. 6) and of the complement activator cobra venom factor[7,8]. This molecule has the capacity to cause fixation of complement on human unbilical vein endothelial cells. We show that complement fixation is a potent and rapid stimulus for neutrophil adhesion. Neutrophil adhesion requires only endothelial deposition of C3, and is mediated through the type 3 complement receptor.**

A hybrid of *Ulex europaeus* I (Ulex) and cobra venom factor (CVF) was constructed using the heterobifunctional cross-linking compound *N*-succinimidyl 3-(2-pyridyldithio)propionate. The hybrid molecule, referred to as Ulex–CVF was radioiodinated for tracing purposes and shown to retain the Ulex lectin property of time and dose saturable binding to human umbilical vein endothelial cells, as well as retaining one third of the native CVF complement-activating activity, as assessed by a haemolytic assay (not shown). Pretreating endothelial cells with Ulex–CVF followed by serum as a complement source induced surface deposition of complement. Endothelial-bound iC3b, C5, and the membrane attack complex (MAC) were detected by radioimmunoassay (Fig. 1).

Treating endothelial cells with Ulex–CVF followed by serum resulted in a high degree of neutrophil adhesion. In Fig. 2 the time and dose relationships for the induction of adhesion are demonstrated. The effect was detected within 1 min of adding the complement source and a high proportion of the cells added could be induced to adhere (approaching 100%). The adhesion reached a peak within 20 min and was maintained for up to 8 hours. Removing the source of complement led to the rapid decay of adherence (not shown).

The time course of adhesion contrasts strongly with that caused by treating endothelial cells with the cytokine tumor necrosis factor α (TNF), a previously defined mechanism for inducing neutrophil-endothelial adhesion[9]. Endothelial cells stimulated with TNF at a concentration of 200 U ml^{-1}, (human recombinant, 1 ng = 10 U, Cetus) had only basal levels of adhesion after 30 minutes stimulation (77% of control versus 1,869% for Ulex-CVF and serum treated cells), a minimal increase after 1 h of stimulation (143% of control adhesion) and only developed a marked increase at 2 h after stimulation (408% of control adhesion). There is also an interesting contrast between the strength of the adhesion induced by complement and other adhesion mechanisms. Using the same washing conditions to remove unbound neutrophils we could barely detect the adhesion commonly studied by activating neutrophils with

TABLE 1 Effect of antibodies to complement receptors and related molecules on neutrophil adhesion to complement-treated endothelium

Antibody	Reference	Specificity	Adhesion inhibition (%)
3D9	21	CR1	0
1B4	21	CR1	0
57F	22	CR1	0
17aba	23	CR3 α (CD11b)	71
44aac	23, 24	CR3 α	75
MN-41	25	CR3 α	65
KB90	26	CR4 α (CD11c)	5
L29	26	CR4 α	0
SPV-L7	27	LFA1 α (CD11a)	0
SPV-L11	27	LFA1 α	0
IB4	28	β-chain (CD18)	89
10F12		β-chain	78

Human umbilical vein endothelial cells were exposed to Ulex–CVF (1.76 μg ml^{-1}) for 2 h at 4 °C followed, after washing, by 80% human serum for 30 min at 37 °C, as described in Fig. 1. Antibody was added to the endothelial cells in 50 μl-phosphate-buffered saline (PBS) followed by another 50 μl containing 2×10^5 ^{51}Cr-labelled neutrophils. Neutrophil preparation and assessment of adhesion were as described in Fig. 2. Results are expressed as % inhibition of adhesion calculated as adhesion with antibody subtracted from adhesion without antibody and divided by adhesion without antibody. Isotype controls had no effect on adhesion and none of the antibodies had any effect on adhesion in the absence of endothelial complement deposition (not shown). The specificity of the antibodies is described in the references given in column two. 10F12 is a common β-chain (CD18) antibody (J. Ritz, personal communication). IB4 does not inhibit binding of iC3b coated erythrocytes to monocytes but it does block this interaction with neutrophils (S. D. Wright, personal communication). All antibodies were in the form of ascites and were used at 1/50 dilution.

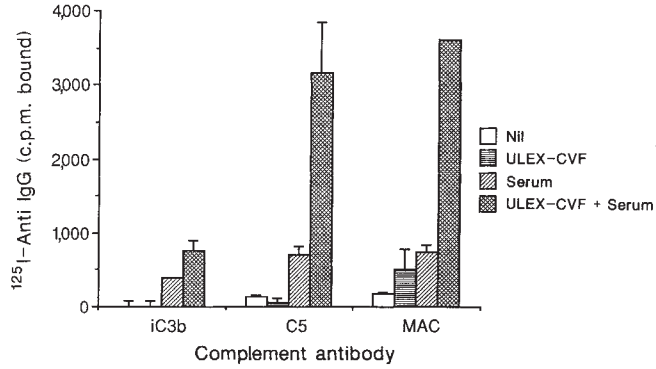

FIG. 1 Induction of endothelial complement deposition by Ulex–CVF. The use of the surface bound complement activator Ulex–CVF followed by human serum as a complement source was associated with the formation of iC3b in the endothelial membrane and binding of C5 and the membrane attack complex. The use of serum alone was associated with some complement fixation but always less than that associated with use of the activator.
METHODS. Endothelial cells were extracted and cultured from human umbilical veins according to the method of Jaffe[29]. Cells were cultured in 20% fetal bovine serum (Hyclone) in medium 199 (Gibco) supplemented with 100 μg ml^{-1} endothelial cell growth supplement (Collaborative Research) and 100 μg ml^{-1} bovine lung heparin (Sigma). Cells used in this and other experiments were derived from the second to fourth passages. Endothelial cells were seeded at 10,000 cells per 6.4 mm diameter microwell (Corning) previously coated with 10 μg ml^{-1} fibronectin (Calbiochem) for 10 min and allowed to grow to confluence for at least 2 days before being used. Confluent monolayers of cells in 96 well plates were washed and exposed to 50 μl of 1.76 μg ml^{-1} Ulex-CVF or diluent alone for 2 h at 4 °C. The wells were washed 3 times with 100 μl volumes and then 100 μl of 80% fresh normal human serum or diluent alone was added for 30 min at 37 °C. The wells were washed 3 times and then either a mouse monoclonal antibody to a human iC3b neoantigen (Cytotech) or rabbit antibodies to human C5 (Calbiochem), or the membrane attack complex (MAC, Calbiochem) added for 1 h at 4 °C. The wells were washed 3 times and then either ^{125}I-labelled F_{ab_2} fragments of sheep anti-mouse immunoglobulin antibody (622 ng ml^{-1}, 9 Ci g^{-1} DuPont) or donkey anti-rabbit immunoglobulin antibody (144 ng ml^{-1}, 6 Ci g^{-1}, Amersham) were added for 1 h at 4 °C. The wells were washed 7 times and residual radioactivity released with 10% SDS and counted. The medium used for dilution and washing was phosphate buffered saline with calcium and magnesium (Gibco) and 0.5% w/v human serum albumin (Cutter) (PBS–HSA), supplemented with 0.02% sodium azide after the addition of antibodies. The results are presented as the mean c.p.m. associated with the use of each antibody minus the c.p.m. associated with use of the appropriate isotype or serum control. Error bars, standard deviation.

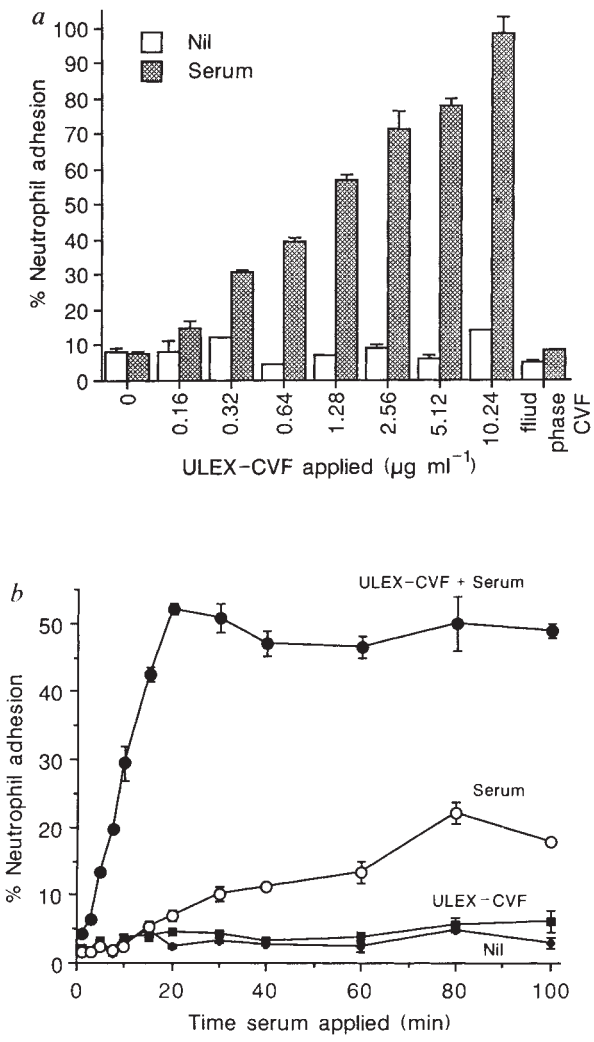

FIG. 2 Induction of neutrophil adhesion by endothelial complement deposition. *a*, Increasing neutrophil adhesion with increasing concentrations of Ulex-CVF followed by the addition of serum as a complement source. The ^{125}I-labelled Ulex-CVF was assessed over a 64-fold range of concentration. Fluid phase CVF refers to the addition of free CVF (Cytotech) at 10 U ml^{-1} with serum, as opposed to endothelial bound CVF, and indicates that fluid phase CVF is not able to substitute for surface bound CVF. *b*, Increasing neutrophil adhesion with increasing serum exposure time. There is some neutrophil adhesion with the use of serum alone, an effect variably present in other experiments, but much greater adhesion when the cells were pretreated with Ulex-CVF. In these and other experiments Ulex-CVF alone had no adhesive effect.

METHODS. Neutrophils were isolated by density gradient centrifugation (endoxotin free Lymphoprep, Nycomed) of citrated whole blood followed by two cycles of hypotonic lysis of the erythrocyte pellet. The final preparation was >95% polymorphonuclear and >95% viable. Neutrophils were labelled with 100 μCi of ^{51}sodium chromate (DuPont) in PBS without calcium or magnesium (Gibco) with gentle shaking for 1 h at 37 °C, washed 3 times with PBS without calcium or magnesium and 0.1% HSA. Radioactivity remained >90% cell associated when assessed at the end of the adhesion assay. Endothelial cells in microwells (prepared as described in Fig. 1) were treated with PBS–HSA containing *a*, increasing amounts of Ulex-CVF or *b*, 1.76 μg ml^{-1} Ulex-CVF for 2 h at 4 °C followed by 3 washes with PBS–HSA. Eighty per cent fresh human serum in PBS–HSA or PBS–HSA alone was then added, incubated with the cells at 37 °C, and removed by washing 3 times with PBS–HSA after *a*, 30 min or *b*, at various intervals between 1 and 100 min. After removing the serum 2×10^5 ^{51}Cr-labelled neutrophils in 100 μl of PBS were added for 15 min at 37 °C. Unattached cells were then removed by 3 washings of 150 μl of PBS, and the radioactivity associated with the bound neutrophils was released with 10% SDS and counted. Percentage adhesion was calculated by dividing the adherent counts by the total counts added per well. ^{125}I-labelled Ulex-CVF did not register any radioactivity when counting ^{51}Cr to assess adhesion. Error bars, standard deviation.

phorbol myristate acetate, formyl-Met-Leu-Phe or calcium ionophore A23187. In further studies, neutrophils maintained in continuous motion were arrested by and bound to complement treated endothelium but did not bind to contiguous control endothelium (not shown), suggesting that this adhesion may occur with sufficient avidity to be relevant to blood flow through the microvasculature.

The neutrophil receptor responsible for adherence to the complement treated endothelium was identified using a range of monoclonal antibodies specific for individual receptors and related molecules (Table 1). Unequivocal results identifying the type 3 complement receptor (CR3) as the responsible molecule were obtained. Antibodies to the α-chain of CR3 (CD11b) as well as antibodies to the β-chain (CD18) it shares with the other CD11 molecules[10] strongly inhibited adhesion. Neither antibodies to the other CD11 molecules (CD11a, CD11c) nor antibodies to CR1 inhibited adhesion.

The finding that neutrophil CR3 was responsible for the binding suggested that iC3b, the only known complement factor which binds CR3 (ref. 11), was the endothelial-bound, ligand. This was investigated using purified complement components and reconstituting the alternate pathway to the extent necessary to induce neutrophil adhesion. It was found that factors B, D and C3 were all necessary and adequate for reconstitution. C3 was used at a concentration equivalent to 17% of that found in normal serum and was associated with a degree of adhesion similar to that caused by 25% serum (Fig. 3). Addition of later complement components as well as the regulatory components H and I led to no further increase in adhesion (not shown). Although recent work has suggested that neutrophil CR3 also binds fibrinogen[12] and coagulation factor X (ref. 13) in addition to iC3b, the reconstitution experiments demonstrated that the complement products were responsible for inducing adhesion. They further indicated that induction of adhesion was independent of the membrane attack complex and any associated injury. Complement factors B, D and C3 and magnesium in the presence

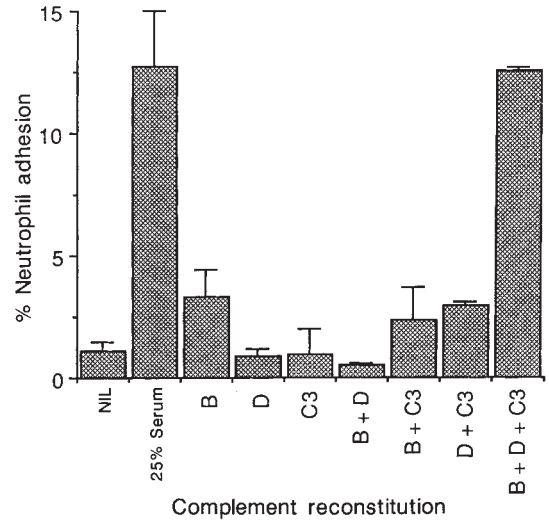

FIG. 3 Reconstitution of neutrophil adhesion with purified complement components B, D and C3 in comparison with the effect of serum. Cells in microwells were treated with PBS–HSA containing 1.76 μg ml^{-1} Ulex-CVF for 2 h at 4 °C followed by 3 washes with PBS–HSA. Then either 25% fresh human serum or isolated complement components (Cytotech) were added in 40 μl of PBS–HSA for 30 min at 37 °C. After 3 washes with PBS–HSA, neutrophils were added and adhesion assessed as in Fig. 2. All complement components were shown to migrate as a single species of the appropriate molecular weight on nonreducing SDS polyacrylamide gel electrophoresis. The concentrations used were B, 100 μg ml^{-1}; D, 1 μg ml^{-1}; C3, 200 μg ml^{-1}. C3 was mixed with the other factors immediately before use. Neither serum nor the complement factors when used in the absence of Ulex-CVF had any effect on neutrophil adhesion (not shown). Error bars, standard deviation.

of an activator result in the formation of C3b (ref. 14). Further studies with ^{125}I-labelled C3, B and D, which assessed the formation of C3b and iC3b by following changes in molecular weight of the C3 α-chain with gel electrophoresis, demonstrated formation of iC3b from C3b (not shown). Although we cannot exclude possible factor I contamination of the isolated factors as a cause of iC3b formation, endothelial cells have detectable transcripts for factors H and I (ref. 15) and it seems likely that endothelium has the endogenous capacity to form iC3b from C3b.

Recent work has demonstrated that cytokine-stimulated endothelium becomes adhesive for leukocytes[9,16], a process requiring synthesis of membrane proteins such as ELAM-1 (ref. 17) and ICAM-1 (ref. 18). Adhesion is not detected until considerably after endothelial stimulation and peaks at ~4 h (ref. 9 and unpublished results). The rapidity with which adhesion may occur *in vivo* in response to various stimuli[2-4] suggests that another mechanism preceding the more slowly developing cytokine-mediated induction of neutrophil-endothelial adhesion may play a role in acute inflammation. Our work demonstrates an additional and novel mechanism by which neutrophil adhesion to the vessel wall may be mediated. Vascular complement deposition in experimental and clinical inflammatory conditions is commonly observed[19]. We suggest that complement activation occurring in the context of an acute inflammatory stimulus adjacent to a vessel wall may cause endothelial complement deposition. It is also possible that an alteration in the susceptibility of the vessel wall to the fixation and subsequent degradation of complement may occur. The mechanism of how this could occur is unclear. It is of interest, however, that the molecule GMP-140, which is related to ELAM-1, is fused into the endothelial plasma membrane very rapidly following an activating stimulus. GMP-140 has sequence homology with proteins that have the common feature of being regulators of complement activation[20].

The important bound complement component is C3 which is converted to iC3b, the ligand of the neutrophil type 3 complement receptor. We noted somewhat less inhibition of adhesion using antibodies to the CR3 α-chain (CD11b) than with antibodies to the β-chain that CR3 has in common with CD11a and CD11c. This suggests at least the possibility of accessory interactions involving these molecules and other ligands. However this does not occur in the absence of prior complement fixation. The important aspects of this mechanism are that it develops rapidly, forms a strong neutrophil-endothelial bond, and retains the endothelial specificity that is such a marked feature of adhesion *in vivo*. □

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1. Florey, H. W. *General Pathology* (Lloyd-Luke, London, 1970).
2. Allison, F., Smith, M. R. & Wood, W. B. *J. exp. Med.* **102,** 655-668 (1955).
3. Atherton, A. & Born, G. V. R. *J. Physiol.* **222,** 447-474 (1972).
4. Björk, J. & Smedegård, G. *Int. Archs. Allergy appl. Immun.* **74,** 178-185 (1984).
5. Cohnheim, J. *lectures on General Pathology* (New Sydenham Society, London, 1889).
6. Holthöfer, H. *et al. Lab. Invest.* **47,** 60-66 (1982).
7. Müller-Eberhard, H. J. & Fjellström, K-E. *J. Immun.* **107,** 1666-1672 (1971).
8. Vogel, C-W., Smith, C. A. & Müller-Eberhard, H. J. *J. Immun.* **133,** 3235-3241 (1984).
9. Gamble, J. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **82,** 8667-8671 (1985).
10. Sanchez-Madrid, F. *et al. J. exp. Med.* **158,** 1785-1803 (1983).
11. Ross, G. D. & Lambris, J. D. *J. exp. Med.* **155,** 96-110 (1982).
12. Wright, S. D. *et al. Proc. natn. Acad. Sci. U.S.A.* **85,** 7734-7738 (1988).
13. Altieri, D. C. & Edgington, T. S. *J. biol. Chem.* **263,** 7007-7015 (1988).
14. Schreiber, R. D., Götze, O. & Müller-Eberhard, H. J. *J. exp. Med.* **144,** 1062-1075 (1976).
15. Ripoche, J. A., *et al. J. exp. Med.* **168,** 1917-1922 (1988).
16. Bevilacqua, M. P. *et al. J. clin. Invest.* **76,** 2003-2011 (1985).
17. Bevilacqua, M. P. *et al. Proc. natn. Acad. Sci. U.S.A.* **84,** 9238-9242 (1987).
18. Smith, C. W. *et al. J. clin. Invest.* **82,** 1746-1756 (1988).
19. McCluskey, R. T. & Fienberg, R. *Hum. Path.* **14,** 305-315 (1983).
20. Johnston, G. I., Cook, R. G. & McEver, R. P. *Cell* **56,** 1033-1044 (1989).
21. O'Shea, J. J. *et al. J. Immun.* **134,** 2580-2587 (1985).
22. Iida, K., Mornaghi, R. & Nussenzweig, V. *J. exp. Med.* **155,** 1427-1438 (1982).
23. Dana, N. *et al. J. Immun.* **137,** 3259-3263 (1986).
24. Arnaout, M. A. *et al. J. clin. Invest.* **72,** 171-179 (1983).
25. Eddy, A. *et al. Clin. Immun. Immunopath.* **31,** 371-389 (1984).
26. McMichael, A. J. *et al. Leucocyte Typing III. White Cell Differentiation Antigens* (Oxford University Press, 1987).
27. Keizer, G. D. *et al. Eur. J. Immun.* **15,** 1142-1147 (1985).
28. Wright, S. D. *et al. Proc. natn. Acad. Sci. U.S.A.* **80,** 5699-5703 (1983).
29. Jaffe, E. A. *Biology of Endothelial Cells.* (Nijhoff, The Hague, 1984).

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Pulsatile intracellular calcium release does not depend on fluctuations in inositol trisphosphate concentration

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MANY hormones, neurotransmitters and growth factors evoke in their target cells oscillations in the free internal Ca^{2+} concentration ($[Ca^{2+}]_i$) (ref. 1). In electrically non-excitable cells these fluctuations are due to periodic release of Ca^{2+} from intracellular reservoirs[1,2], stimulated by the internal messenger inositol trisphosphate ($InsP_3$) (refs 2–4). Most models at present invoke fluctuating levels of $InsP_3$ as a key component in generating the oscillations in $[Ca^{2+}]_i$ (refs 5 and 6). $InsP_3$ injected into intact cells evokes irregular and transient oscillatory Ca^{2+}-dependent current responses[7-9], but the intracellular $InsP_3$ concentration is not constant in such experiments. Here we monitor changes in $[Ca^{2+}]_i$ by measuring Ca^{2+}-activated Cl^- current in single internally perfused mouse pancreatic acinar cells[4,10-12] and show that acetylcholine (ACh), acting through muscarinic receptors, evokes regular and repetitive current pulses which are mimicked by $InsP_3$ applied through a patch pipette[13]. To exclude the possibility that $InsP_3$ is periodically phosphorylated[14] or degraded, we replaced it by the non-metabolizable $InsP_3$ analogue inositol trisphosphorothioate ($InsPS_3$)[9,15,16], which also evokes regular pulses of Ca^{2+}-activated Cl^- current. These effects are independent of external Ca^{2+}, but abolished by high intracellular concentrations of a Ca^{2+}-chelator. We conclude that repetitive pulses of intracellular Ca^{2+} release occur even when the concentration of $InsP_3$ is constant.

Figure 1*a* shows that 0.1 μM ACh evokes regular pulses of inward current. In this experiment there were approximately 3 pulses per min, although there was variation from cell to cell (2–6 per min) and the duration of the pulses ranged from ~3–18 s. This pattern was obtained in nine experiments. In one experiment there was no ACh effect whereas in two other experiments a sustained non-oscillating response was observed. The frequency range of the stimulant-evoked current pulses at room temperature is similar to that of the $[Ca^{2+}]_i$ fluctuations observed in intact mouse pancreatic acinar cells[17] (3–4 per min, at 30 °C), parotid acinar cells[18] (6–16 per min, at room temperature), liver cells[19] (1 per 2 min–2 per min, at 37 °C) and neutrophils[20] (1–2 per min, at 37 °C). At 1 μM, ACh normally evoked an oscillating response, as seen in Fig. 1*b*, but the current did not return to the baseline between the peaks. At 10 μM, ACh evoked a smooth sustained increase in the inward current (Fig. 1*c*). All effects of ACh were abolished by atropine (Fig. 1*a*, *c*) ($n = 13$) as well as by including a high concentration of the Ca^{2+}-chelator EGTA in the pipette filling solution (Fig. 1*d*) ($n = 5$). The effect of ACh was independent of the presence or absence of external

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