under investigation.

The number of MHC-antigen complexes required to stimulate T cells is likely to vary not only with the APC source, but with the T cell population as well. The affinity of the T cell receptor for the antigen-MHC complexes is undoubtedly one important factor in determining the number of complexes required for T cell activation. The T cell hybridomas used in this and other studies have been very often selected for their high reactivity with antigen, and therefore the number of antigen-MHC complexes required to activate these cells may differ from the requirements for normal T cell activation.

REFERENCES AND NOTES

1. S. Demotz, H. M. Grey, E. Appella, A. Sette, *Nature* **342**, 682 (1989).
2. J. S. Spencer and R. T. Kubo, *J. Exp. Med.* **169**, 625 (1989).
3. L. Adorini *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5181 (1988).
4. S. Buus, A. Sette, H. M. Grey, *Immunol. Rev.* **98**, 115 (1987).
5. J. Krieger *et al.*, *J. Immunol.* **140**, 388 (1988).
6. A. Kelso, A. L. Glasebrook, O. Kanagawa, K. T. Brunner, *ibid.* **129**, 550 (1982).
7. N. Shastri, B. Malissen, L. Hood, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5885 (1985).
8. P. Naquet *et al.*, *Immunogenetics* **22**, 247 (1985).
9. H. M. Grey, S. M. Colon, R. W. Chesnut, *J. Immunol.* **129**, 2389 (1982).

10. We thank L. Adorini for IE5 cells, M. Albertson and S. Edelman for technical assistance, and J. Joseph for secretarial assistance. Supported by NIH grants AI 09758 and AI 18634 and a fellowship from Schweizerische Stiftung fuer Medizinisch-Biologische Stipendien (to S.D.).

10 April 1990; accepted 12 July 1990

Adhesion of Human B Cells to Germinal Centers in Vitro Involves VLA-4 and INCAM-110

ARNOLD S. FREEDMAN, J. MICHAEL MUNRO, G. EDGAR RICE, MICHAEL P. BEVILACQUA, CHIKAO MORIMOTO, BRADLEY W. MCINTYRE, KURT RHYNHART, JORDAN S. POBER, LEE M. NADLER

Human B lymphocytes localize and differentiate within the microenvironment of lymphoid germinal centers. A frozen section binding assay was developed for the identification of those molecules involved in the adhesive interactions between B cells and lymphoid follicles. Activated human B cells and B cell lines were found to selectively adhere to germinal centers. The VLA-4 molecule on the lymphocyte and the adhesion molecule INCAM-110, expressed on follicular dendritic cells, supported this interaction. This cellular interaction model can be used for the study of how B cells differentiate.

THE GERMINAL CENTER OF THE LYMPHOID follicle is a specialized microenvironment that participates in the proliferation and differentiation of B lymphocytes. B lymphocytes enter lymphoid organs by first binding to high endothelial venules via specific adhesion receptors and subsequently migrating between the endothelial cells into the stroma (*1–4*). In the lymphoid organ they proliferate within the germinal center and then differentiate into either memory or immunoglobulin-producing cells (*5*). The major constituents of germinal centers are activated B cells, but follicular dendritic cells (FDC) as well as some macrophages and T cell subsets are also located there (*6, 7*). Within the germinal center, activated B cells are physically associated with the FDCs, but the precise functional interactions between B cells and other cellular components of the germinal center are unknown (*8*).

It is presently unknown how B cells localize within follicles or their germinal centers. Since the interaction of lymphocytes with high endothelial venules was characterized by preferential binding to endothelial cells in frozen tissue sections (*9*), we reasoned that a similar assay might be developed to show adhesion of B cells to follicles or their germinal centers. Highly purified human splenic B cells (>95% $CD20^+$) that were either unstimulated or cultured for 3 days with *Staphylococcus aureas* Cowan Strain I (SAC) were internally labeled with the fluorochrome sulfofluorescein diacetate (SFDA) to permit their visualization when bound to particular areas of tissue sections. The B cells cultured in SAC were determined to be activated by their increased cell size and expression of several B cell activation antigens (*10–12*). When activated B cells were incubated at 25°C on frozen sections of human tonsil, they adhered preferentially to germinal centers, as shown by combined fluorescence and bright-field microscopy (Fig. 1A and Table 1). Rare cells binding to extrafollicular areas, including microvessels, were also seen. Binding of SAC-stimulated cells to the germinal centers was essentially absent at 4°C. Unstimulated B cells showed little to no binding to germinal centers at either 25° or 4°C (Fig. 1B and Table 1). This result suggests that following activation, B cells undergo changes in cell surface antigens that facilitate their retention in germinal centers.

Considering the well-recognized adhesion between leukocytes and endothelial cells observed in frozen section binding assays, we examined whether the binding of B cells to germinal center regions could be explained on the basis of such an interaction. Sections were stained immunohistochemically with monoclonal antibody (MAb) to von Willebrand factor for identification of endothelial cells (Fig. 1C). Scattered capillaries were present within germinal centers, but their distribution was insufficient to account for the pattern and extent of B cell binding. This result indicates that activated B cells were adhering to structures other than endothelium within the germinal center.

Lymphoid cell lines with distinct cell surface phenotypic characteristics and endothelial binding properties have been useful in the identification of a number of homing receptors (*13, 14*). We examined expression

Table 1. Binding of resting and stimulated B cells to germinal centers. Resting and stimulated splenic B cells were used in the binding assay as described (Fig. 1) for 25 min at 25°C. Slides were independently examined blindly. Cells were counted on fluorescence photomicrographs, and germinal center areas were assessed on bright-field photomicrographs of the same sections with a Zeiss VideoPlan digitizing board. Splenic B cells from three individuals were examined for their binding to tonsillar sections from three different individuals and expressed as mean ± SEM number of cells bound per 0.1 mm^2 of germinal center area for three different tonsils.

Donors	Lymphocytes bound/0.1 mm^2	
	Resting B	Stimulated B
1	0.4 ± 0.2	235.0 ± 31.9
2	0.3 ± 0.3	212.9 ± 12.9
3	1.9 ± 0.5	145.7 ± 49.4

A. S. Freedman, C. Morimoto, K. Rhynhart, L. M. Nadler, Division of Tumor Immunology, Dana-Farber Cancer Institute and Department of Medicine, Harvard Medical School, Boston, MA 02115.
J. M. Munro, G. E. Rice, M. P. Bevilacqua, J. S. Pober, Departments of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115.
B. W. McIntyre, Department of Immunology, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030.

of known cell-surface adhesion molecules on four B cell lines to determine whether there was a relationship between antigen expression and germinal center binding (Table 2). The Nalm-6 cell line (*15*), which avidly bound to germinal centers, expressed CD54 (ICAM-1) (*16*), CD58 (LFA-3) (*17*), and CDw49d (VLA α_4)/CD29 (VLA β) (*18*). However, three other cell lines, which exhibited less binding, similarly expressed these antigens. This finding suggests that although these adhesion molecules may be necessary, their expression is insufficient to explain binding of Nalm-6 to germinal centers. Nalm-6 cells did not express CD11a/CD18 (LFA-1 α/β) (*17, 19*), CD44 (Hermes antigen) (*2, 14, 20*), or LAM-1/Leu 8 (MEL-14 homolog) (*21*), whereas the other cell lines were variably positive, suggesting that these lymphocyte adhesion molecules were not involved in the observed binding.

To determine which, if any, of the lymphocyte cell surface adhesion molecules expressed by Nalm-6 were involved in germinal center binding, we examined a panel of MAbs for inhibition of Nalm-6 binding. The adhesion of Nalm-6 to germinal centers was blocked by antibody to CD29 (anti-CD29) and antibody to CDw49d (anti-CDw49d), whereas antibody to CD54 (anti-CD54), antibody to CD58 (anti-CD58), or antibody to CD19 (anti-CD19) had no statistically significant effect (Table 3). The germinal center binding of normal SAC-stimulated B cells was similarly blocked by anti-CD29 and anti-CDw49d MAbs (*22*). Additional MAbs to cell surface antigens expressed on Nalm-6 including HLA-DR (MAb 279), MHC class I (W6/32), CD9 (J2), CD10 (J5), CD24 (HB8), CD38 (HB7), and CD71 (T9) did not affect germinal center binding. The antibody to CD33 (anti-CD33) [immunoglobulin G2b (IgG2b) isotype MAb], which is unreactive with Nalm-6, was used as an isotype-identical negative control for anti-CDw49d MAb and did not affect binding.

To further clarify that VLA-4 was involved in germinal center binding, we examined Nalm-6 cells for expression of the other VLA α chains (*18, 23*). Only VLA α_4 (CDw49d) was detected by indirect immunofluorescence, whereas MAbs to VLA α_1, α_2, α_3, α_5, and α_6 were unreactive with Nalm-6 (*24*).

Our data showed that normal activated B cells and Nalm-6 cells bound to either cells or extracellular matrix constituents that are specific to the germinal center. A likely cellular candidate would be the FDC, which is unique to the germinal center and is known to associate with activated B cells both in vivo and in vitro (*8*). The inducible cell adhesion molecule (INCAM)–110, originally identified on cytokine-activated endothelium, mediates the adhesion of human melanoma cells as well as isolated human lymphocytes and monocytes (*25, 26*). Immunohistochemical studies of human tissues revealed that follicular dendritic cells reacted with a MAb directed against INCAM-110 (anti–INCAM-110) (*26*). Moreover, we have shown that isolated human FDCs also express INCAM-110 (*27*). When anti–INCAM-110 was incubated on tissue sections, no significant germinal center binding of Nalm-6 cells was seen (Table 3). In contrast, treatment of tissue sections with anti-CD54, which reacts strongly with FDCs or anti-CD19, showed minimal inhibition of Nalm-6 binding. However, in oth-

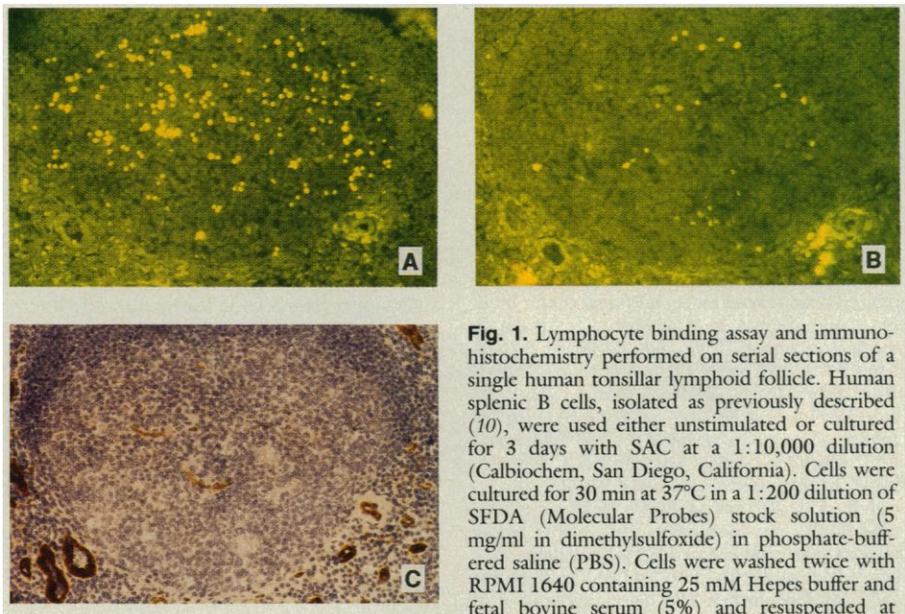

Fig. 1. Lymphocyte binding assay and immunohistochemistry performed on serial sections of a single human tonsillar lymphoid follicle. Human splenic B cells, isolated as previously described (*10*), were used either unstimulated or cultured for 3 days with SAC at a 1:10,000 dilution (Calbiochem, San Diego, California). Cells were cultured for 30 min at 37°C in a 1:200 dilution of SFDA (Molecular Probes) stock solution (5 mg/ml in dimethylsulfoxide) in phosphate-buffered saline (PBS). Cells were washed twice with RPMI 1640 containing 25 mM Hepes buffer and fetal bovine serum (5%) and resuspended at 3×10^7 cells/ml. Immediately before the assay, clumps of cells were removed by passing the cell suspension through nylon mesh. The cell suspension (100 µl) was placed onto two 8-µm frozen sections of tonsil within a 2.2-cm-diameter circle delineated by a ring of 12 M dimethylpolysiloxane, rotating at 70 revolutions per minute for 25 min at 25°C. After incubation, slides were fixed in 3% glutaraldehyde (Polysciences, Warrington, Pennsylvania) in PBS overnight at 4°C. Sections were rinsed, stained with hematoxylin, then photographed sequentially under bright-field and fluorescent illumination. (**A**) Fluorescently labeled stimulated B cells. (**B**) Fluorescently labeled unstimulated B cells. (**C**) Parallel section immunostained with a MAb to von Willebrand factor (Dako, Copenhagen, Denmark) (avidin-biotin method with diaminobenzidine) (*26*).

Table 2. Expression of cell surface adhesion molecules and germinal center binding of B cell lines. Cell lines were examined by indirect immunofluorescence and flow cytometric analysis with MAbs (2F12, anti-CD11a; 10F12, anti–CD18; 515, anti-CD44; RR1/1, anti-CD54; TS2/9, anti-CD58; anti-LAM-1; 4B4, anti-CD29; and L25, anti-CDw49d), all of murine γ1 isotype except anti–LAM-1 (μ) and L25 (γ2b), detected with goat antibody to mouse immunoglobulin coupled to fluorescein isothiocyanate (FITC). Cell surface antigen expression was determined by the mean peak channel (−, ≤10; +, 10 to 85; ++, 86 to 170; and +++, 171 to 256) on a three-log scale with an EPICS C flow cytometer (Coulter Electronics, Hialeah, Florida). Cell lines were used in the adhesion assay previously described (Fig. 1). The binding assay is representative of one of three experiments with three different specimens of tonsil and expressed as mean ± SEM number of cells bound per 0.1 mm² of germinal center area. Ag, antigen.

Cell line	Germinal center binding (cells/0.1 mm²)	Cell surface antigen expression						
		CD54 (ICAM-1)	CD58 (LFA-3)	CD29 (VLA β)	CDw49d (VLA α_4)	CD11α/18 (LFA-1 α/β)	CD44 (HERMES Ag)	LAM-1 (Leu 8)
Nalm-6	78.8 ± 25.4	+	++	++	+	−	−	−
Raji	13.0 ± 1.4	+++	++	++	+	++	−	++
Cess	5.2 ± 1.4	++	++	++	+	+++	+++	++
Jijoye	4.2 ± 2.4	+++	++	++	+	−	+++	−

er experiments no significant effect of anti-CD19 and anti-CD54 MAbs on Nalm-6 binding was observed.

The induction of immunoglobulin synthesis, heavy chain class switching, affinity selection, and the generation of memory B cells depends on complex intercellular interactions within the germinal center (*6, 28*). In an in vitro binding assay, activated B cells preferentially adhered to the germinal center, and VLA-4 is one of the ligands involved in lymphoid cell binding. However, the expression of VLA-4 is not sufficient to explain germinal center binding, since a number of VLA-4–positive cell lines did not bind. This suggests that associated structures may be required to confer specific germinal center adhesion and may include cell surface molecules that are induced with activation. This has been postulated for LPAM-1 and LPAM-2, which mediate murine-lymphocyte binding to Peyer's patch HEV. The expression of these molecules, whose α chain is homologous to VLA-4, is not sufficient for HEV binding (*3*). Alternatively, an activation-associated change in the avidity of VLA-4 for its ligand may be required for binding, analogous to that seen with CD11a/18 (*29*). The enhanced adhesion of activated B cells to germinal centers may be due to either a qualitative or quantitative change in VLA-4 expression, and further studies may provide insight into this issue.

A cDNA has been isolated from activated endothelial cells that encodes a molecule designated VCAM-1 (*30*). This member of the immunoglobulin supergene family was shown to mediate the adhesion of certain lymphoid cell lines, suggesting a relationship to INCAM-110. It has now been shown that anti–INCAM-110 MAb binds to COS cells transfected with a partial VCAM-1 cDNA (*31*). It has also been shown that VLA-4, in addition to functioning as a fibronectin receptor (*32*), is a receptor for VCAM-1 (*33*). Therefore, B lymphocyte binding to germinal centers is likely to be the result of a direct interaction between VLA-4 on lymphoid cells and INCAM-110 expressed on FDC.

Most B cell leukemias and lymphomas are thought to be neoplastic counterparts of normal activated B cells (*11, 34*). The clinical behavior of these malignancies reflects the ability of tumor cells to localize and proliferate within specific microenvironments. Specifically, follicular lymphomas, which account for at least 50% of non-Hodgkin's lymphomas, recapitulate normal lymphoid germinal centers (*34*). This study provides a means to further our understanding of normal B cell differentiation, as well as a context within which to study the interaction of neoplastic B cells with a specific microenvironment.

Table 3. Effect of antibody treatment of Nalm-6 cells and tissue sections on germinal center binding. Nalm-6 cells were incubated in media or media containing MAbs (B4, B43H12, B410F, anti-CD19; 4B4, anti-CD29; RR1/1, W-CAM-1, anti-CD54; TS2/9, BRIC 5, anti-CD58; L25, anti–VLA α_4; and E1/6, anti-INCAM 110), all murine γ1 isotypes, except MY9, W-CAM-1, and L25, which are murine γ2b, and BRIC 5, which is murine γ2a, used at saturating binding concentrations for 20 min at 25°C. Cells were then washed and resuspended in fresh media before application to tissue sections. Tissue sections were similarly treated with MAbs in PBS for 20 min at 25°C, then washed in PBS before application of Nalm-6 cells. These data represent one of three experiments with three different specimens of tonsil and expressed as mean ± SEM number of cells bound per 0.1 mm^2 of germinal center area.

MAb (cell line)	No. of germinal center binding cells/0.1 mm^2
Cell treatment	
Media	131.5 ± 37.9
Media +	
Anti-CD19 (B4)	187.8 ± 28.8
Anti-CD19 (B43H12)	149.2 ± 22.1
Anti-CD19 (B410F)	105.4 ± 19.9
Anti-CD54 (RR1/1)	171.3 ± 29.0
Anti-CD54 (W–CAM-1)	161.0 ± 21.0
Anti-CD58 (TS2/9)	118.2 ± 16.5
Anti-CD58 (BRIC 5)	205.7 ± 20.7
Anti-CD33 (MY9)	198.2 ± 48.4
Anti-CD29 (4B4)	0.5 ± 0.3
Anti-CDw49d (L25)	0.6 ± 0.4
Tissue section treatment	
Media	146.4 ± 21.8
Media +	
Anti-CD19 (B4)	89.3 ± 10.6
Anti-CD54 (RR1/1)	84.2 ± 7.3
Anti–INCAM-110 (E1/6)	0.5 ± 0.5

REFERENCES AND NOTES

1. J. L. Gowans and E. J. Knight, *Proc. R. Soc. London B Biol. Sci.* **159**, 257 (1964); A. O. Anderson and N. D. Anderson, *Immunology* **31**, 731 (1976).
2. I. Stamemkovic, M. Amiot, J. M. Pesando, B. Seed, *Cell* **56**, 1057 (1989); L. A. Goldstein *et al.*, *ibid.*, p. 1063.
3. B. Holzmann, B. W. McIntyre, I. L. Weissman, *ibid.*, p. 37; B. Holzmann and I. L. Weissman, *EMBO J.* **8**, 1735 (1989).
4. L. A. Lasky *et al.*, *Cell* **56**, 1045 (1989); M. H. Siegelman, M. Van De Rijn, I. L. Weissman, *Science* **243**, 1165 (1989); P. R. Streeter, E. Lakey Berg, B. T. N. Rouse, R. F. Bargatze, E. C. Butcher, *Nature* **331**, 41 (1988).
5. G. J. Thorbecke, R. Asofsky, G. M. Hochwald, G. W. Siskind, *J. Exp. Med.* **116**, 295 (1962).
6. E. C. Butcher *et al.*, *J. Immunol.* **129**, 2698 (1982).
7. G. Gutman and I. Weissman, *Immunology* **23**, 465 (1972); R. V. Rouse, J. A. Ledbetter, I. Weissman, *J. Immunol.* **128**, 2243 (1982); S. Fossum and W. L. Ford, *Histopathology* **9**, 469 (1985); D. N. J. Hart and J. L. McKenzie, *J. Exp. Med.* **168**, 157 (1988); P. Nieuwenhuis and W. L. Ford, *Cell. Immunol.* **23**, 254 (1976).
8. G. J. V. Nossal, A. Abbot, J. Mitchell, Z. Lummus, *J. Exp. Med.* **127**, 277 (1968); P. Nieuwenhuis and F. J. Keuning, *Immunology* **26**, 509 (1974); E. Heinen *et al.*, *Eur. J. Immunol.* **14**, 267 (1984); K. Inaba, M. D. Witmer, R. M. Steinman, *J. Exp. Med.* **160**, 858 (1984); C. T. Schnizlein, M. H. Kosco, A. K. Szakal, J. G. Tew, *J. Immunol.* **134**, 1360 (1985); A. K. Szakal, M. H. Kosco, J. G. Tew, *ibid.* **140**, 341 (1988); F. Schriever *et al.*, *J. Exp. Med.* **169**, 2043 (1989).
9. H. B. Stamper and J. J. Woodruff, *J. Exp. Med.* **144**, 828 (1976); E. C. Butcher *et al.*, *J. Immunol.* **123**, 1996 (1979); L. M. Stoolman and S. D. Rosen, *J. Cell Biol.* **96**, 722 (1983).
10. A. W. Boyd *et al.*, *J. Immunol.* **134**, 1516 (1985).
11. A. S. Freedman *et al.*, *Leukemia* **1**, 9 (1987).
12. Indirect immunofluorescence and flow cytometric analysis of SAC-stimulated splenic B cells showed that 50 to 70% of cells were CD25$^+$ and greater than 90% were CD71$^+$.
13. E. C. Butcher, R. G. Scollay, I. L. Weissman, *Eur. J. Immunol.* **10**, 556 (1980); W. M. Gallatin, I. L. Weissman, E. C. Butcher, *Nature* **304**, 30 (1983).
14. S. T. Jalkanen, R. F. Bargatze, L. R. Herron, E. C. Butcher, *Eur. J. Immunol.* **16**, 1195 (1986).
15. J. Minowada *et al.*, *J. Natl. Cancer Inst.* **60**, 1269 (1978).
16. R. Rothlein, M. L. Dustin, S. D. Marlin, T. A. Springer, *J. Immunol.* **137**, 1270 (1985).
17. F. Sanchez-Madrid *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7489 (1982).
18. M. E. Hemler, C. Huang, L. Schwarz, *J. Biol. Chem.* **262**, 11478 (1987); M. E. Hemler *et al.*, *ibid.*, p. 3300; C. Morimoto *et al.*, *J. Immunol.* **134**, 3762 (1985).
19. D. Davignon, E. Martz, T. Reynolds, K. Kurzinger, T. A. Springer, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4535 (1981).
20. L. J. Picker, J. De Los Toyos, M. J. Telen, B. F. Haynes, E. C. Butcher, *J. Immunol.* **142**, 2046 (1989).
21. T. F. Tedder *et al.*, *J. Exp. Med.* **170**, 123 (1989); D. Camerini, S. P. James, I. Stamenkovic, B. Seed, *Nature* **342**, 78 (1989); T. F. Tedder, A. C. Penta, H. B. Levine, A. S. Freedman, *J. Immunol.* **144**, 532 (1990).
22. A. Freedman, K. Rhynhart, L. M. Nadler, unpublished observations.
23. Y. Fradet *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 224 (1984); F. Sanchez-Madrid *et al.*, *Eur. J. Immunol.* **16**, 1344 (1986); A. Sonnenberg, H. Janssen, F. Hogervorst, J. Calafat, J. Hilgers, *J. Biol. Chem.* **262**, 10376 (1987); K. D. Pischel, M. E. Hemler, C. Huang, H. G. Bluestein, V. L. Woods, *J. Immunol.* **138**, 226 (1987).
24. By indirect fluorescence and flow cytometric analysis, Nalm-6 was unreactive with the following MAbs: TS2/7 (anti-VLA α_1), 12F1 (anti-VLA α_2), J143 (anti-VLA α_3), BIE5 (anti-VLA α_5), and GOH3 (anti-VLA α_6).
25. G. E. Rice and M. P. Bevilacqua, *Science* **246**, 1303 (1989).
26. G. E. Rice, J. M. Munro, M. P. Bevilacqua, *J. Exp. Med.* **171**, 1369 (1990).
27. M. Munro, A. Freedman, L. M. Nadler, unpublished observations.
28. G. Kraal, I. L. Weissman, E. C. Butcher, *Nature* **298**, 377 (1982); G. Kraal, R. R. Hardy, W. M. Gallatin, I. L. Weissman, E. C. Butcher, *Eur. J. Immunol.* **16**, 829 (1986); Y. J. Liu *et al.*, *Nature* **342**, 929 (1989).
29. M. L. Dustin and T. A. Springer, *Nature* **341**, 619 (1989).
30. L. Osborn *et al.*, *Cell* **59**, 1203 (1989).
31. M. P. Bevilacqua and A. Aruffo, unpublished observation.
32. E. A. Wayner, A. Garcia-Pardo, M. J. Humphries, J. A. McDonald, W. G. Carter, *J. Cell Biol.* **109**, 1321 (1989); J. L. Guan and R. O. Hynes, *Cell* **60**, 53 (1990).
33. M. J. Elices *et al.*, *Cell* **60**, 577 (1990).
34. E. S. Jaffe, E. M. Shevach, M. M. Frank, C. W. Berard, I. Green, *N. Engl. J. Med.* **290**, 814 (1974); R. B. Mann *et al.*, *ibid.* **295**, 685 (1976); H. Stein, J. Gerdes, D. Y. Mason, *Clin. Haematol.* **11**, 531 (1982); J. Cossman, L. M. Neckers, T. Jones, S. M. Hsu, E. S. Jaffe, *Am. J. Pathol.* **115**, 117 (1984); N. L. Harris, L. M. Nadler, A. K. Bhan, *ibid.*, p. 262.

35. We thank S. F. Schlossman, R. A. Rasmussen, E. L. Reinherz, and T. F. Tedder for critical review of the manuscript. We also thank the following investigators for providing MAbs: T. F. Tedder for anti–LAM-1, B43H12, and B410F; J. Ritz for 2F12 and

10F12; T. A. Springer for RR1/1 and TS2/9; M. Hemler for TS2/7 (anti-VLA α_1); V. Woods for 12F1 (anti-VLA α_2); L. Old for J143 (anti-VLA α_3); C. Damsky for BIE5 (anti-VLA α_5); A. Sonnenberg for GOH3 (anti-VLA α_6); G. S. Kansas for 515 (anti-CD44); and A. W. Boyd for W-CAM-1. BRIC 5 was provided from the 4th International Workshop on Leukocyte Differentiation Antigens. We appreciate the assistance of K. Hildebrandt for preparation of the manuscript. Supported by NIH grants CA40216, AR33713, 5T32HL07627-03, PO1 HL36028, and HL07727; PHS grant CA-01105 (to A.S.F.); American Cancer Society grant IM-549 and Texas Advanced Research Program grant 15-071 (to B.W.M.); and the American Heart Association (to J.S.P.).

14 February 1990; accepted 22 May 1990

Molecular Cloning and Functional Expression of Glutamate Receptor Subunit Genes

JIM BOULTER, MICHAEL HOLLMANN, ANNE O'SHEA-GREENFIELD, MELISSA HARTLEY, EVAN DENERIS,* CORNELIA MARON, STEPHEN HEINEMANN

Three closely related genes, GluR1, GluR2, and GluR3, encode receptor subunits for the excitatory neurotransmitter glutamate. The proteins encoded by the individual genes form homomeric ion channels in *Xenopus* oocytes that are sensitive to glutamatergic agonists such as kainate and quisqualate but not to *N*-methyl-D-aspartate, indicating that binding sites for kainate and quisqualate exist on single receptor polypeptides. In addition, kainate-evoked conductances are potentiated in oocytes expressing two or more of the cloned receptor subunits. Electrophysiological responses obtained with certain subunit combinations show agonist profiles and current-voltage relations that are similar to those obtained in vivo. Finally, in situ hybridization histochemistry reveals that these genes are transcribed in shared neuroanatomical loci. Thus, as with γ-aminobutyric acid, glycine, and nicotinic acetylcholine receptors, native kainate-quisqualate–sensitive glutamate receptors form a family of heteromeric proteins.

THE AMINO ACID L-GLUTAMATE ACTS as an excitatory neurotransmitter at many synapses in the mammalian central nervous system. Glutamate is involved in fast excitatory synaptic transmission (*1*), the regulation of neurotransmitter release (*2*), long-term potentiation, learning and memory (*3*), developmental synaptic plasticity (*4*), hypoxic-ischemic damage and neuronal cell death (*5*), epileptiform seizures (*6*), as well as the pathogenesis of several neurodegenerative disorders (*7*). This functional diversity is reflected by complex electrophysiology and pharmacology in which glutamate acts via disparate receptors and conductance mechanisms (*8*). Glutamate receptor classification schemes are based on pharmacological criteria that define five receptor subtypes or classes: receptors gating cation-selective ion channels that are activated by *N*-methyl-D-aspartic acid (NMDA), kainic acid (KA), α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) (formerly called the quisqualic acid or QUIS receptor), or 2-amino-4-phosphonobutyric acid (AP4), and the metabotropic receptor activated by 1-amino-cyclopentyl-1,3-dicarboxylic acid (ACPD).

The relation between the KA and AMPA receptor subtypes and the channels they gate is uncertain. It is not known if KA and AMPA activate the same receptor-channel complex with different efficacies or if distinct receptor-channel complexes exist for these agonists (*9*). Since no specific antagonists are available that distinguish KA- from AMPA-evoked responses, the existence of two non-NMDA receptor subtypes has been inferred from the differential potency of specific agonists. Results from [^{3}H]KA and [^{3}H]AMPA binding studies have been cited as evidence both for (*10*) and against (*11*) the hypothesis of distinct KA and AMPA receptor subtypes. However, definitive conclusions are difficult to make because of the uncertain relation between binding sites and the effector sites for depolarization.

To investigate the relation between structure and function of the various glutamate receptor subtypes we have applied molecular biological techniques. As a first step, we used an expression cloning approach to isolate a cDNA clone, GluR1 (*12*), which encodes a 99,800-dalton protein capable of forming a homomeric, KA-gated ion channel in *Xenopus* oocytes (*13*). Our initial results suggested that the KA responses measured in oocytes injected with in vitro synthesized GluR1 RNA or rat brain polyadenylated [poly(A$^+$)] RNA were indistinguishable. We have now tested whether the GluR1 gene is a member of a larger, glutamate receptor subunit gene family and whether the proteins encoded by these related genes function as subunits of glutamate receptors other than the KA subtype.

Complementary DNA clones encoding the GluR2 (λRB14) and GluR3 (λRB312) genes were isolated from an adult rat forebrain library by using a low-stringency hybridization screening protocol and a radiolabeled fragment of the GluR1 cDNA as a probe (*14*) (Fig. 1). The calculated molecular weights of the mature, nonglycosylated forms of GluR2 and GluR3 are 96,400 (862 amino acids) and 98,000 (866 amino acids), respectively. Potential N-linked glycosylation sites occur in the GluR2 protein at Asn^{235}, Asn^{349}, Asn^{385}, Asn^{392}, and Asn^{835} and in the GluR3 protein at Asn^{35}, Asn^{238}, Asn^{352}, Asn^{387}, and Asn^{394}. As for GluR1 (*12*), the hydrophobicity profiles for GluR2 and GluR3 reveal five strongly hydrophobic regions: one such domain is located at the NH_2-terminus of each protein and has characteristics of a signal peptide (*15*), while four additional hydrophobic regions probably form membrane-spanning helices (*16*) (MSR I to IV) (Fig. 1 and below) and are located in the COOH-terminal half of each polypeptide.

Significant sequence identity exists between GluR1 and both GluR2 (70%) and GluR3 (69%) as well as between GluR2 and GluR3 (74%), particularly in the COOH-terminal half of each protein. This region includes the four postulated membrane-spanning regions where, acknowledging five conservative substitutions, there is complete sequence identity. An unexpected finding is the extensive sequence identity (allowing conservative substitutions, 98%) in the segment between MSR III and IV. In other ligand-gated ion channels this region, modeled to be part of a cytoplasmic loop, shows the highest intersubunit variation in both length and sequence.

To test whether the GluR2 and GluR3 proteins function as homomeric, KA-sensitive ion channels, we injected oocytes with RNA transcripts synthesized in vitro from individual cDNA clones (*17*). Both GluR2- and GluR3-injected oocytes depolarized in response to bath application of 100 μM KA (Fig. 2A). However, the amplitudes of the KA responses were not equivalent for the glutamate receptor subunits: with equal amounts of injected RNA (2 ng), responses in GluR3 RNA-injected oocytes were invariably larger than GluR1 responses. KA-

Molecular Neurobiology Laboratory, The Salk Institute for Biological Studies, San Diego, CA 92138.

*Present address: Center for Neurosciences, School of Medicine, Case Western Reserve University, Cleveland, OH 44106.