distinct advantages, including an ability to label most cells in a population for long periods of time and the potential to investigate the synaptic connectivity of transplanted neurons through the identification of colloidal gold–containing nerve terminals by means of electron microscopy. In light of recent clinical trials involving the intracerebral transplantation of dopamine-secreting cells to treat Parkinson's disease (*14*) and basic research exploring the possible use of cholinergic transplants to treat Alzheimer's disease (*15*), it would be advantageous to have a labeling technique capable of monitoring the viability and development of transplanted neuronal tissues. The technique described here could be useful clinically in neural transplants and could be helpful in understanding the plasticity and connectivity of transplanted neural tissues.

REFERENCES AND NOTES

1. A. Fine, *Sci. Am.* **255**, 52 (August 1986).
2. A. Bjorklund and U. Stenevi, Eds., *Neural Grafting in the Mammalian CNS* (Elsevier, Amsterdam, 1985).
3. G. D. Das and J. Altman, *Science* **173**, 637 (1971); R. D. Lund and S. D. Hauschka, *ibid.* **193**, 582 (1976); G. D. Das and J. Altman, *Brain Res.* **38**, 233 (1972); G. D. Das, *TIT J. Life Sci.* **4**, 93 (1974); S. K. McConnell, *Science* **229**, 1268 (1985); E. G. Jones and M. K. Floeter, in (*2*), pp. 217–233.
4. Y. Okada, *Exp. Cell Res.* **26**, 98 (1962); A. Loyter, N. Zakai, R. G. Kulka, *J. Cell Biol.* **66**, 292 (1975).
5. D. J. Volsky and A. Loyter, *FEBS Lett.* **92** (no. 2), 190 (1978).
6. Approximately 50 to 100 ml of the virus-containing Sendai (Cantell) hemagglutinin are centrifuged at 3000 rpm for 20 minutes and the pellet is discarded. The supernatant is recentrifuged at 37,000*g* for 30 minutes at 4°C and the supernatant discarded. The pellet is then resuspended in 1 ml of solution 1 (100 m*M* NaCl and 50 m*M* tris-base, *p*H 7.4) containing 1 μl of a 10^{-5}*M* stock solution of phenylmethylsulfonylfluoride and 10 μl of a 20% stock solution of Triton X-100. The mixture is left at 20°C for 1 hour, then centrifuged at 100,000*g* for 1 hour at 4°C. The pellet is discarded as it contains viral nucleocapsids. The supernatant is retained and colloidal gold solution is added v/v, followed by dialysis (12,000 molecular weight pore size) against 1 g of Bio-beads SM-2 in 900 ml of distilled water plus 100 ml of solution 2 stock [10 m*M* tris-base, 2 m*M* $CaCl_2$, and 2 m*M* $MgCl_2$ (*p*H, 7.4) to a final volume of 0.5 liter] for 5 hours at 25°C and then for 30 to 35 hours at 4°C. The dialysate is centrifuged at 100,000*g* for 5 minutes at 4°C, and the pellet is resuspended in 1 ml of solution 1. The gold-encapsulated envelopes can be stored at 9°C for several weeks. Colloidal gold preparation consisted of the following: 2.5 ml of 1% AuCl, 15 ml of 1% sodium citrate, and 232.5 ml of water. Boil water, add citrate, and boil vigorously. Then add 2.5 ml of 1% AuCl and reflux for 15 minutes. Store mixture in a sterile dark brown bottle at 9°C. To concentrate colloids, centrifuge at 100,000*g* for 5 minutes at 4°C. Retrieve the soft pellet in a minimal volume. To dilute colloids, add solution 1 to desired volume (usually 1 ml).
7. G. W. Arendash, P. N. Strong, P. R. Mouton, in *Neurology and Neurobiology*, J. T. Hutton and A. D. Kenny, Eds. (Liss, New York, 1985), vol. 18, pp. 351–376; A. Bjorklund, U. Stenevi, R. H. Schmidt, S. B. Dunnett, F. H. Gage, *Acta Physiol. Scand. Suppl.* **522**, 1 (1983).
8. We found that a 10% v/v suspension of cells to viral envelopes was appropriate for labeling, with no more than 10 μl of cells in any one incubation tube (1 ml of cell suspension contains approximately 20,000 cells). Thus, for 10 μl of cell suspension, 100 μl of viral envelopes was added. However, each preparation of envelopes may vary. Solution 1 plus 10 m*M* $CaCl_2$ was added to the cell suspension and viral envelopes to make a final volume of 1 ml in each tube. The mixture was then incubated with moderate shaking at 37°C for 1 hour. After incubation, the solution was centrifuged at 750 rpm for 1 minute at 4°C and the supernatant discarded. This washing was repeated several times with solution 1, followed by a final centrifugation at 750 rpm for 1 minute at 4°C and removal of the supernatant. The colloidal gold–labeled cells can then be resuspended in an appropriate buffer. We resuspended them in a 0.9% NaCl, 0.6% glucose solution and transplanted the cells as soon as possible.
9. With the incisor bar set at 2.8 mm below the interaural line, coordinates for the four cell suspension infusions in each animal were as follows: 1.5 mm posterior to bregma (P), 2.0 and 4.0 mm lateral to midline (L), and 1.6 and 1.8 mm vertical (V), respectively; and at 3.5 mm P, 2.0 and 4.0 mm L, and 1.6 and 1.8 mm V, respectively.
10. Recipient rats, weighing 280 to 295 g, were anesthetized with sodium pentobarbital (50 mg/kg) and infused with 1 μl of ibotenic acid (5 μg/ml) at two sites within the nBM by means of the stereotaxic atlas of J. Konig and A. Klippel [*The Rat Brain: A Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem* (Williams & Wilkins, Baltimore, 1963)]. With the incisor bar set at 2.8 mm below the interaural line, infusion coordinates were: 7.0 mm A (anterior to the interaural line), 2.6 mm L, and 6.5 and 5.7 mm V.
11. Animals were perfused with 10% neutral buffered Formalin. Their brains were removed and placed in 10% Formalin for several days before frozen sectioning at 30 to 40 μm. All brain sections were counterstained with Mayer's acid hemalum (40 seconds) for visualization of both labeled and unlabeled cell bodies.
12. C. W. Cotman, N. Nieto-Sampedro, S. R. Whittemore, in (*2*), pp. 169–178; G. A. Banker, *Science* **209**, 809 (1980); H. W. Muller and W. Seifert, *J. Neurosci. Res.* **8**, 195 (1982).
13. R. Lindsay, C. Emmett, G. Raisman, P. Seeley, *Ann. N.Y. Acad. Sci.* **495**, 35 (1987); C. Sotelo and R. Alvarado-Mallart, *ibid.*, p. 242; M. Gumpel, F. Lachapelle, C. Jacque, N. Maumann, in (*2*), pp. 151–158.
14. I. Madrazo *et al.*, *N. Engl. J. Med.* **316**, 831 (1987).
15. G. W. Arendash, P. N. Strong, P. R. Mouton, in *Neurology and Neurobiology*, J. T. Hutton and A. D. Kenny, Eds. (Liss, New York, 1985), vol. 18, pp. 351–376; S. B. Dunnett *et al.*, *Neuroscience* **16**, 787 (1985); F. H. Gage, A. Björklund, U. Stenevi, S. B. Dunnett, P. A. T. Kelly, *Science* **225**, 533 (1984).

16. Supported by a grant from the Alzheimer's Disease and Related Disorders Association, NIH grant HD 17933 (G.A.), and a University of South Florida Division of Sponsored Research grant (A.M.). We thank V. Kriho for provocative conversations leading to our development of this technique and acknowledge the photographic processing expertise of F. P. Olivero, University of South Florida staff photographer.

11 August 1987; accepted 18 November 1987

Cells Process Exogenous Proteins for Recognition by Cytotoxic T Lymphocytes

JONATHAN W. YEWDELL,* JACK R. BENNINK,* YASUHIRO HOSAKA

Cells exposed to intact, noninfectious influenza virus were shown to be recognized by class I–restricted anti-influenza cytotoxic T lymphocytes (CTLs). Both internal and external proteins derived from virions were processed by cells for CTL recognition. Sensitization required the inactivation of viral neuraminidase activity and could be inhibited by preventing fusion of viral and cellular membranes. These findings are important in designing vaccines to elicit CTL responses, since they demonstrate that cells can process intact, exogenous proteins for recognition by CTLs and suggest that such processing depends on introduction of exogenous proteins into the cytoplasm.

INFLUENZA VIRUS HAS BEEN USED EXtensively to study the recognition of foreign antigens by cytotoxic T lymphocytes (CTLs) restricted by class I molecules of the major histocompatibility complex (MHC) [reviewed in (*1*)]. Seven viral proteins are present in virions (*2*). Two integral membrane proteins, hemagglutinin (HA) and neuraminidase (NA), are embedded in the host-derived lipid envelope. Matrix protein (M1) is believed to form a subenvelope shell enclosing the ribonucleoprotein (RNP) core, which consists of viral RNA complexed with nucleoprotein (NP), and small amounts of three viral polymerases (PA, PB1, and PB2). Viral replication is initiated by delivery of the RNP to the host cell cytoplasm. The HA mediates two functions essential to this process. First, it attaches virus to the cell by binding sialic acid residues present on cellular glycoproteins and glycolipids. Second, after internalization of virus into cellular endosomes, the subsequent acidification triggers conformational alterations in the HA that lead to the fusion of viral and cellular membranes (*3, 4*). Although the NA does not function directly in penetration, its ability to cleave sialic residues prevents irreversible HA-mediated attachment of virions to macromolecules or nonendocytosing cells (*5*).

Early studies showed that de novo synthesis of viral proteins was required for CTL-mediated lysis of influenza virus–infected

J. W. Yewdell and J. R. Bennink, Wistar Institute for Anatomy and Biology, Philadelphia, PA 19104.
Y. Hosaka, Department of Preventive Medicine, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565 Japan.

*Present address: Laboratory for Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892.

target cells. Incubation of cells with noninfectious intact virions, or intact proteins derived from virions, failed to sensitize cells for CTL recognition (*6*). More recently, it was found that the requirement for de novo synthesis of viral proteins could be circumvented by incubating cells with fragments of viral proteins, including oligopeptides consisting of as few as 11 amino acid residues (*7*). These findings have led to the idea that CTL recognition of cells exposed to foreign proteins is dependent on the alteration of the protein by cellular biosynthetic or catabolic pathways (*8*).

It is uncertain whether the difference in the ability of cells to process exogenous versus endogenously synthesized proteins is related to the process of biosynthesis itself or simply to the intracellular location of newly synthesized proteins. We show that the latter explanation is correct by demonstrating that cells can process exogenous proteins if sufficient quantities of the proteins are introduced into the cytoplasm.

CTL recognition of proteins derived from input virions was examined by incubating L929 cells (H-2^k) with PR8 [A/Puerto Rico/8/34 (H1N1)] irradiated with ultraviolet light to inactivate viral infectivity (UV virus). To ensure that residual noninactivated viral genes did not direct synthesis of viral proteins, we incubated cells in the presence of inhibitors of viral RNA and protein synthesis (*9*). In agreement with the original studies (*6*), anti-influenza CTL populations from CBA mice (H-2^k) failed to specifically lyse cells incubated with UV virus (Table 1, experiment A). Cells were lysed at high levels, however, if UV virus was heated for 30 minutes at 55°C before being added to the cells (Table 1, experiment A). This extends the observation of Hosaka *et al.* (*10*) that CTLs recognize cells exposed to heated virus that is not UV-irradiated.

Our earlier findings indicated that anti-influenza CTLs from H-2^k mice recognize four viral gene products expressed by infected cells (*11*). These include HA, NP, PB1, and nonstructural 1 (NS1), a protein abundantly synthesized early in the infectious cycle and completely excluded from mature virions (*2*). To determine which proteins are recognized on cells sensitized with heated virus, we used secondary CTL populations stimulated in vitro and specific for each of these four proteins. This revealed that both internal (NP and PB1) and external (HA) virion proteins are processed for CTL recognition by target cells (Table 1, experiment A). Even though NS1-specific CTLs lysed infected cells at high levels, they failed to lyse cells sensitized with heated noninfectious virus. This indicates that recognition of heated virus-sensitized cells is not due to protein synthesis from non-inactivated viral genes. Additional evidence for the absence of viral protein synthesis was provided by the failure of NS1-specific monoclonal antibodies (mAbs) to stain fixed and permeabilized heated virus-sensitized cells in indirect immunoperoxidase assays. NS1 was easily detected by immunoperoxidase staining of infected cells in the same experiment.

Heat treatment of PR8 did not alter its cell binding or fusing activities, as determined by hemagglutination and hemolysis assays, respectively. It did, however, greatly reduce NA activity, a finding consistent with the known thermal lability of the N1 subtype NA (*12*). To determine whether NA inactivation was related to target cell sensitization, we incubated virus for various times and temperatures in the presence or absence of Ca^{2+}, an agent that enhances the

Table 1. CTL recognition of individual components from noninfectious virions. L929 cells were trypsinized, washed, and suspended at 2×10^7 cells per milliliter in Eagle's minimum essential medium adjusted to *p*H 6.8 with 25 m*M* Hepes. Cells (100 μl) were incubated with 2000 hemagglutinating units (HAU) of infectious, UV-irradiated, or heated UV-irradiated virus. Virus was prepared by centrifuging freshly harvested infectious allantoic fluid for 2 hours at 19,000 rpm in a Beckman type 19 rotor. Pelleted virus was resuspended in phosphate-buffered saline at a concentration of 2×10^5 HAU/ml and stored at −70°C. Freshly thawed virus was UV irradiated at 250 uW/cm^2 for 10 minutes (experiment A) or 20 minutes (experiment B). UV-irradiated virus was heated at 55°C for 30 minutes (A) or 1 minute (B). In experiment B, DDAN (Boehringer Manheim) was added with UV-irradiated virus to a concentration of 1 m*M*. After 1.5 hours at 37°C, cells were diluted to 10^6 per milliliter with RPMI 1640 supplemented with 5% fetal bovine serum, incubated for 2 hours at 37°C, and labeled for 1 hour at 37°C with $Na^{51}CrO_4$. Effector cells were splenocytes from CBA mice, primed with PR8 or vaccinia virus recombinants containing cloned PR8 genes encoding HA, NP, PB1, or NS1, stimulated in vitro for 6 days with autologous PR8-infected splenocytes. Spenocytes derived from recombinant vaccinia virus–primed mice stimualted in this manner demonstrate exclusive anti-influenza CTL specificity for the influenza virus gene product expressed by the vaccinia virus recombinant. Effector cells were incubated with target cells at a ratio of 10:1 for 4 hours at 37°C, and the amount of ^{51}Cr released into the supernatant was determined by gamma counting. Specific ^{51}Cr release is defined as experimental release minus spontaneous release (no CTL) divided by total release (in the presence of detergent) minus spontaneous release.

L929 target cells	Specific ^{51}Cr release (%) with CTLs specific for				
	HA	NP	PB1	NS1	PR8
	Experiment A				
Uninfected	5	11	6	7	6
Infectious PR8	69	81	81	71	77
UV PR8	14	16	8	7	11
UV, 55°C PR8	46	71	77	10	64
	Experiment B				
Uninfected	9	13	11		
Infectious PR8	46	86	80		
UV PR8	12	14	12		
UV, 55°C PR8	64	43	52		
UV PR8 + 1 m*M* DDAN	79	42	52		
Uninfected + 1 m*M* DDAN	12	16	11		

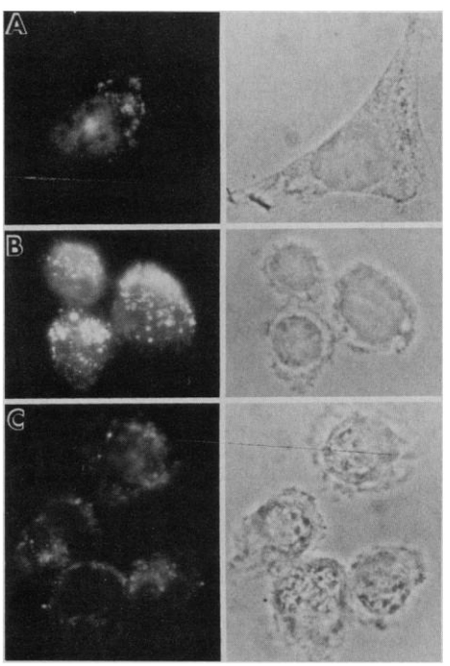

Fig. 1. Immunofluorescence of target cells sensitized with heated virus. Cells sensitized with heated virus were stained by indirect immunofluorescence (left) and monoclonal antibodies specific for NP (**A** and **C**) or M1 (**B**). Corresponding fields were visualized with phase-contrast optics (right). In (**C**), cells were continuously incubated in the presence of 25 m*M* NH_4Cl. L929 cells were prepared and incubated with heated virus for 1 hour as described in Table 1. Cells were then suspended at 4×10^5 cells per milliliter in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 10^{-5} *M* cycloheximide and added to cleaned glass cover slips (12-mm diameter) placed in 24-well tissue culture plates (500 μl per well). After 9 hours of incubation at 37°C, cells were fixed for 20 minutes with 3% paraformaldehyde and permeabilized for 2 minutes with 1% Triton X-100. Cells were then stained with monoclonal antibodies as described (*19*).

thermal stability of the NA (*12*). Virus incubated in the absence of Ca^{2+} at 37°C for 60 minutes, or at 55°C for 3 minutes, lost at least 99% of its NA activity (as determined by cleavage of sialic acid from fetuin) and sensitized target cells for CTL lysis (Table 2). By contrast, virus heated under the same conditions in the presence of Ca^{2+}, or incubated at 0°C for 60 minutes in the absence of Ca^{2+}, maintained NA activity and failed to sensitize target cells (Table 2). Virus heated for as little as 1 minute at 55°C in the absence of Ca^{2+} effectively sensitized target cells (Table 1, experiment B).

To test the relation between target cell sensitization and viral NA inactivation, we incubated nonheated UV virus with L929 cells in the presence of a reversible NA inhibitor, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (DDAN) (*13*). Threshold sensitization was observed with concentrations as low as 10 μ*M*, and reached maximum levels at 1 m*M* (Table 1, experiment B). Sensitization was not caused by DDAN-induced alterations in target cells, since (i) sensitization did not occur in the absence of virus (Table 1, experiment B) and (ii) the antigen-specific nature of recognition was preserved, as determined by the failure of NS1-specific CTLs to lyse sensitized cells in additional experiments.

These data indicate that inactivation of viral NA activity facilitates the cellular processing of viral components into CTL recognition structures. Two mechanisms may account for this phenomenon. NA inactivation might alter the manner in which cells handle input virus. Alternatively, NA inactivation might simply increase the amount of virus available for cellular processing by preventing the elution of virus from cellular sialic acid receptor molecules. Indirect immunofluorescence staining of fixed and permeabilized cells sensitized with heated virus is consistent with the latter possibility; staining of cells incubated with nonheated virus by monoclonal antibodies specific for NP, HA, or M1 was barely detectable, whereas cells sensitized with heated virus were intensely stained (Fig. 1).

This experiment revealed the presence of NP in the nucleus of heated virus-sensitized cells (in contrast, HA and M1 were detected only in the cytoplasm). Taking into account the karyophilic properties of NP (*14*), this finding demonstrates that heated virus fuses with target cells and releases its RNP core into the cytoplasm. The importance of viral fusion activity to target cell sensitization was tested in two ways. First, coincident with the addition of heated virus to cells, cells were continuously incubated in the presence of NH_4Cl. NH_4Cl prevents the acidification of cellular endosomes and inhibits the fusion process (*4, 15*). This treatment prevented the migration of NP to the nucleus (Fig. 1C) and abrogated CTL recognition (Table 2). NH_4Cl-mediated inhibition was not due to inhibition of cellular processing as such, since CTL recognition was not affected if viral penetration was allowed to proceed for 30 minutes prior to the addition of NH_4Cl. Second, inactivation of viral fusion activity by brief exposure of heated virus to *p*H 5 (*16*) prevented both the nuclear migration of NP and target cell sensitization (Table 2).

Taken together with the observation that cleavage of HA into disulfide-linked subunits activates viral fusion activity in parallel with the ability of heated virus to sensitize target cells (*10*), these findings indicate that fusion of viral and cellular membranes is an essential step in the cellular processing of virion proteins for CTL recognition.

Further events in the processing of virion-derived proteins await definition. It is possible that integral membrane proteins such as the HA, which presumably remain associated with cellular membranes after fusion, are processed by a different pathway than internal proteins like NP, which are released into the cytoplasm. Further, the degree to which processing pathways for exogenous proteins and proteins synthesized de novo overlap must also be established.

More than a decade has elapsed since the importance of viral fusion activity was established in CTL recognition of cells sensitized with noninfectious Sendai virus, a paramyxovirus that fuses with the plasma membrane of cells at neutral *p*H (*17*). Our results generalize this finding to viruses that fuse with cellular membranes only after internalization into acidic endosomes. Further, they provide the initial indication that both internal and external viral proteins are processed for CTL recognition by cells that have fused with virions. The fact that all types of virion proteins are recognized after fusion, including even minor components such as PB1, suggests that inactivated viruses that maintain fusion activity might represent optimal vaccines for eliciting immune responses that include cellular as well as humoral elements. Finally, our results suggest that inactivation of the receptor-destroying activity of viruses (for example, the neuraminidase of ortho- and paramyxoviruses) could enhance their ability to elicit T cell responses.

Table 2. Target cell sensitization correlates with the inactivation of viral NA. Virus prepared as in legend to Table 1 was incubated at various times and temperatures with 3 m*M* EDTA, or with 3 m*M* EDTA and 11 m*M* $CaCl_2$. At the end of the incubation period, heated virus was placed on ice, and $CaCl_2$ was added to calcium-free virus to a final concentration of 11 m*M*. Virus was acid-treated by incubating for 10 minutes at 37°C in the presence of 75 m*M* sodium acetate, *p*H 5. Target cells were prepared as described in Table 1. Cells sensitized with UV-irradiated virus were incubated with biosynthesis inhibitors (*9*). For one group of cells, 25 m*M* NH_4Cl was present during all manipulations, including the ^{51}Cr release assay. Effector cells were splenocytes derived from CBA mice primed with A/Japan/305 (H2N2) stimulated in vitro with PR8-infected autologous cells. NA assays were performed as described (*18*). The percentage of NA activity was determined by parallel titrations of treated and untreated viruses.

L929 target cells	Incubation		Ca^{2+}	NA activity (%)	Specific ^{51}Cr release (%) with anti-influenza CTLs at	
	Minutes	°C			8:1	3:1
Uninfected					4	2
Infectious PR8	None				99	94
UV PR8	None			100	9	9
UV PR8	60	0	−	100	12	8
UV PR8	60	37	−	1	49	37
UV PR8	60	37	+	100	1	1
UV PR8	3	55	−	<1	85	69
UV PR8	3	55	+	100	9	5
UV PR8	30	55	−	<1	82	71
UV PR8 + *p*H 5	30	55	−	<1	6	2
UV PR8 + NH_4Cl	30	55	−	<1	4	4

REFERENCES AND NOTES

1. G. L. Ada and P. D. Jones, *Curr. Top. Microbiol. Immunol.* **128**, 1 (1987); J. W. Yewdell and C. J. Hackett, in *The Influenza Viruses*, R. Krug, Ed. (Plenum, New York, in press).
2. R. A. Lamb, in *Genetics of Influenza Viruses*, P. A. Palese and D. W. Kingsbury, Eds. (Springer-Verlag, New York, 1983), p. 21.
3. T. Maeda, K. Kawasaki, S. I. Ohnishi, *FEBS Lett.* **122**, 283 (1980); J. J. Skehel *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 968 (1982).
4. K. Matlin, H. Reggio, H. Helenius, K. Simons, *J. Cell Biol.* **91**, 601 (1981).
5. P. Palese, K. Tobita, M. Ueda, R. W. Compans, *Virology* **61**, 397 (1974).
6. T. J. Braciale and K. L. Yap, *J. Exp. Med.* **147**, 1246 (1978); U. H. Koszninowski, H. Allen, M.-J. Gething, M. Waterfield, H.-D. Klenk, *ibid.* **151**, 945 (1980); H. Ertl and G. L. Ada, *Immunobiology* **158**, 239 (1981); L. A. Morrison, A. E. Lukacher, V. L. Braciale, D. P. Fan, T. J. Braciale, *J. Exp. Med.* **163**, 903 (1986).
7. M. Wabuke-Bunoti, D. P. Fan, T. J. Braciale, *J. Immunol.* **127**, 1122 (1981); A. Yamada, M. R. Ziese, J. F. Young, Y. K. Yamada, F. A. Ennis, *J. Exp. Med.* **162**, 663 (1985); A. R. M. Townsend, F.

M. Gotch, J. Davey, *Cell* **42**, 13 (1985); A. R. M. Townsend *et al.*, *ibid.* **44**, 959 (1986).
8. R. N. Germain, *Nature (London)* **322**, 687 (1986).
9. Emetine ($10^{-5}M$) and actinomycin D ($4 \times 10^{-6}M$) were included in all media used prior to the actual ^{51}Cr release assay; cycloheximide ($10^{-5}M$) was present during the assay.
10. Y. Hosaka, F. Sasao, R. Ohara, *Vaccine* **2**, 245 (1985).
11. J. R. Bennink, J. W. Yewdell, G. L. Smith, B. Moss, *J. Virol.* **57**, 786 (1986).
12. D. Bucher and P. Palese, in *The Influenza Viruses and Influenza*, E. D. Kilbourne, Ed. (Academic Press, New York, 1975), p. 84.
13. P. Meindl, G. Bodo, J. Linder, P. Palese, *Z. Naturforsch. Teil B* **26**, 792 (1971).
14. J. Davey, N. J. Dimmock, A. Colman, *Cell* **40**, 667 (1985).
15. A. Yoshimura *et al.*, *J. Virol.* **43**, 284 (1982).
16. J. W. Yewdell, W. Gerhard, T. Bachi, *ibid.* **48**, 239 (1983).
17. J. Schrader and G. M. Edelman, *J. Exp. Med.* **145**, 523 (1977); U. Koszinowski, M.-J. Gething, M. Waterfield, *Nature (London)* **267**, 160 (1977).
18. G. Russ, E. Voreukova, B. Styk, *Acta Virol.* **18**, 299 (1974).
19. T. Bachi, W. Gerhard, J. W. Yewdell, *J. Virol.* **55**, 307 (1985).
20. This work was supported by NIH grants AI-22114 and AI-14162.

13 October 1987; accepted 31 December 1987

Mitogenesis in Response to PDGF and Bombesin Abolished by Microinjection of Antibody to PIP_2

KOOZI MATUOKA, KIYOKO FUKAMI, OSAMU NAKANISHI, SADAAKI KAWAI, TADAOMI TAKENAWA

The turnover of phosphatidylinositol 4,5-bisphosphate (PIP_2) is believed to constitute a crucial step in the signaling pathways for stimulation of cells by a variety of bioactive substances, including mitogens, but decisive evidence for the idea has not been obtained. In the present study, a monoclonal antibody to PIP_2 was microinjected into the cytoplasm of NIH 3T3 cells before or after exposure to mitogens. The antibody completely abolished nuclear labeling with [^{3}H]thymidine induced by platelet-derived growth factor and bombesin, but not by fibroblast growth factor, epidermal growth factor, insulin, or serum. The findings strongly suggest that PIP_2 breakdown is crucial in the elicitation and sustaining of cell proliferation induced by some types of mitogens such as platelet-derived growth factor and bombesin.

MUCH ATTENTION HAS BEEN PAID to inositol phospholipids recently because of their crucial, although so far indecisive, roles in transmembrane signal transduction (*1*). Treatment of cells with mitogens such as platelet-derived growth factor (PDGF) and bombesin rapidly elicits an enzymatic hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (*2*), both intracellular second messengers (*1, 3*). Moreover, inositol phospholipid turnover has been reported to be enhanced in cells transformed by tumor viruses and chemical carcinogens (*4*). PIP_2 breakdown is therefore believed to trigger cell proliferation. However, direct evidence to prove this has not been provided. In addition, mitogens such as fibroblast growth factor (FGF) and insulin do not elicit PIP_2 breakdown (*5–7*). Epidermal growth factor (EGF) is also considered not to evoke PIP_2 breakdown (*6, 7*) except in some cells such as A431 epidermoid carcinoma cells (*8*). These findings raise the question of whether PIP_2 breakdown is an indispensable process for the promotion of cell proliferation. Our aim in the present study, in which an antibody to PIP_2 was microinjected, was to establish the presumed involvement of PIP_2 breakdown in the signaling pathways of cell proliferation. We showed that inhibition of PIP_2 breakdown by the antibody abolishes the stimulation of quiescent cells caused by some types of mitogen.

For development of the antibody to PIP_2, BALB/c mice were immunized with PIP_2 from bovine spinal cords, and hybridoma cells were prepared by a conventional method (*9*). Among the cells, a clone secreted an antibody of the immunoglobulin G2b class. The immunoglobulin G (IgG) produced by this clone (designated antibody kt3g) was purified and examined for its affinity for lipids (Fig. 1a). Treatment with antibody kt3g resulted in a pattern identical with that of a control (treated with IgG from an unimmunized mouse), except for stained PIP_2. Therefore antibody kt3g was found to bind to PIP_2 and to have virtually no specific affinity for other lipids. Such specific binding of antibody kt3g to PIP_2 was further confirmed by an enzyme-linked immunosorbent assay (*10*). The results also showed that antibody kt3g did not cross-react with IP_3 and that it had a weak but detectable affinity for phosphatidylinositol 4-phosphate (PIP). In order to examine effects of antibody kt3g on the turnover of PIP_2 and PIP, we treated a cell membrane preparation with the antibody and determined the changes in the amounts of endogenous PIP_2 and PIP during a subsequent incubation. Antibody kt3g strongly suppressed the decrease in the membranous PIP_2 (Fig. 1b). This effect appeared to depend on the dose of antibody kt3g. Treatment with IgG from an unimmunized mouse had no effect, and antibody kt3g exerted little effect on the turnover of PIP. These results indicate that antibody kt3g specifically interacts with PIP_2 in the membrane and suppresses its turnover. The reason for the antibody effect on PIP_2 turnover is not clear, but it is conceivable that binding of antibody kt3g to PIP_2 would mask the reaction site for phospholipase C, the key enzyme for PIP_2 turnover (*1*), and this would lead to interference with the action of phospholipase C on PIP_2. Indeed, the hydrolysis of PIP_2 in an in vitro assay system using purified phospholipase C (*11*) was decreased by 92.4 ± 2.4% after preincubation of PIP_2 with one-fourth the equivalent of antibody kt3g, whereas antibody kt3g reduced the hydrolysis of PIP in a parallel assay only by 18.1 ± 12.0% (*12*). Therefore, it is likely that antibody kt3g introduced into cells by microinjection would also specifically block PIP_2 breakdown.

NIH 3T3 cells (clone 5611 from the Japanese Cancer Research Resources Bank) were inoculated onto glass cover slips at a density of 5×10^3 to 1×10^4 cell/cm^2 in the presence of 10% calf serum. Quiescence was achieved by culturing the cells in a serum-free medium supplemented with transferrin (human, 5 μg/ml) and albumin (bovine serum fraction V, 0.5 mg/ml) for 24 to 36 hours (*13*). Microinjection was then performed as follows. Antibody kt3g and the control IgG were prepared as solutions (0.1 to 5 mg/ml) in phosphate buffer (140 m*M* K^+, *p*H 7.25) and injected into the cytoplasm of cells with an injectoscope (Olympus, model IMT-2-SYF) (*14*). An attempt was made to inject all of the cells in certain compartments on cover slips delimited by scratches of a diamond pen. The cells were washed and incubated for 2 hours in the medium described above. After addition of mitogens, the cells were cultured in the presence of [^{3}H]thymidine (1 μCi/ml) for the indicated period and sampled for autoradiography (*15*) to determine the labeling indices (the percentages of the cells that incorporated [^{3}H]thymidine) of both the injected and uninjected cells.

K. Matuoka, K. Fukami, O. Nakanishi, T. Takenawa, Department of Pharmacology, Tokyo Metropolitan Institute of Gerontology, Tokyo, 173 Japan.
S. Kawai, Department of Tumor Virus Research, Institute of Medical Science, University of Tokyo, Tokyo, 108 Japan.