mined by scintillation spectroscopy, and the concentration of phosphate in the labeling medium, which was ~0.25 mM. We derived the estimate of 1.5 mol of phosphate per mole of protein by assuming that the specific activity of phosphate in protein was the same as that in the labeling medium. This value probably underestimates the extent of phosphorylation.
20. G. L. Wilson, C. H. Fox, A. S. Fauci, J. H. Kehrl, *J. Exp. Med.* **173**, 137 (1991).
21. I. Stamenkovic and B. Seed, *Nature* **345**, 74 (1990).
22. B. Dorken *et al.*, *J. Immunol.* **136**, 4470 (1986).
23. A. Pezzutto, P. S. Rabinovitch, B. Dorken, G. Moldenhauer, E. A. Clark, *ibid.* **140**, 1791 (1988).
24. A. Pezzutto, B. Dorken, G. Moldenhauer, E. A. Clark, *ibid.* **138**, 98 (1987).
25. I. Stamenkovic, D. Sgroi, A. Aruffo, M. S. Sy, T. Anderson, *Cell* **66**, 1133 (1991).
26. I. Stamenkovic, D. Sgroi, A. Aruffo, *ibid.* **68**, 1003 (1992).
27. M. L. Dustin and T. A. Springer, *Nature* **341**, 619 (1989).
28. G. Di Minno *et al.*, *Blood* **61**, 140 (1983).
29. J. Lesley *et al.*, *J. Exp. Med.* **175**, 257 (1992).
30. M. P. Kamps and B. M. Sefton, *Oncogene* **2**, 305 (1988).
31. ———, *Anal. Biochem.* **176**, 22 (1989).
32. W. H. Fischer, D. Karr, B. Jackson, M. Park, W. Vale, *Methods Neurosci.* **6**, 69 (1991).
33. A. Pezzuto, B. Dörken, G. Moldenhauer, P. S. Rabinovitch, E. A. Clark, in *Leukocyte Typing III*, A. J. McMichael, Ed. (Oxford Univ. Press, Oxford, 1987), pp. 361–364.
34. We thank M. Park for technical assistance. Supported by a fellowship from Schering-Plough (M.-A.C.); by the Clayton Foundation for Research, California Division (W.H.F.); and by grants CA 14195 and CA17289 from the National Cancer Institute of the U.S. Public Health Service.

17 June 1992; accepted 2 September 1992

# A Surface Protease and the Invasive Character of Plague

Ola A. Sodeinde,* Y. V. B. K. Subrahmanyam, Kimberly Stark,† Thomas Quan, Youdi Bao,‡ Jon D. Goguen§

**A 9.5-kilobase plasmid of *Yersinia pestis*, the causative agent of plague, is required for high virulence when mice are inoculated with the bacterium by subcutaneous injection. Inactivation of the plasmid gene *pla*, which encodes a surface protease, increased the median lethal dose of the bacteria for mice by a millionfold. Moreover, cloned *pla* was sufficient to restore segregants lacking the entire *pla*-bearing plasmid to full virulence. Both *pla*$^+$ strains injected subcutaneously and *pla*$^-$ mutants injected intravenously reached high titers in liver and spleen of infected mice, whereas *pla*$^-$ mutants injected subcutaneously failed to do so even though they establish a sustained local infection at the injection site. More inflammatory cells accumulated in lesions caused by the *pla*$^-$ mutants than in lesions produced by the *pla*$^+$ parent. The Pla protease was shown to be a plasminogen activator with unusual kinetic properties. It can also cleave complement C3 at a specific site.**

Three members of the genus *Yersinia* are pathogenic for humans. Two of these, *Y. enterocolitica* and *Y. pseudotuberculosis*, are transmitted by ingestion and generally cause a self-limiting mesenteric lymphadenitis or ileitis. In contrast, *Y. pestis* is usually transmitted by the bite of infected fleas and produces the highly invasive, fulminant, and often fatal disease known as plague. Despite these differences in mode of transmission and the character of the diseases they cause, *Y. pestis* and *Y. pseudotuberculosis* are so closely related that *Y. pestis* is classified as a *Y. pseudotuberculosis* subspecies (*1*). Thus, it is of interest to compare these two organisms and to examine features unique to *Y. pestis* that could be responsible for its high virulence.

The most prominent genetic difference between these two species is the presence of two unique plasmids, 110 and 9.5 kb in size, in *Y. pestis*. Although the influence of the larger plasmid on virulence is not known, the smaller plasmid has been shown to contribute to the invasive character of plague. Loss of this plasmid increases the median lethal dose ($LD_{50}$) of *Y. pestis* for mice by a factor of more than a million, but only when the initial infection occurs at a superficial site (such as in subcutaneous injection) (*2*). If the bacteria are delivered to deeper tissues (by intraperitoneal or intravenous inoculation), little effect on lethal dose is observed. Although it would probably be of no benefit to the orally transmitted yersiniae, the ability to invade deeper tissues after superficial inoculation of the host by flea bite is essential to the flea-rodent-flea life cycle of *Y. pestis* in nature: the bacteria must produce bacteremia to ensure infection of new fleas. Thus, acquisition of the 9.5-kb plasmid was probably an essential step in the evolution of *Y. pestis* from the *Y. pseudotuberculosis* line. The acquisition of this plasmid is also probably responsible for the extreme virulence of *Y. pestis* because death in human plague appears to result from the large numbers of disseminated bacteria rather than from the effects of a specific toxin. The proximal cause of death is most likely reaction of the host to "endotoxin," the lipopolysaccharide component common to the outer membrane of almost all Gram-negative bacteria, including the yersiniae (*3*). Small amounts of this material stimulate host defenses; however, large amounts are lethal, producing high fever, disseminated intravascular coagulation, and irreversible shock.

The 9.5-kb plasmid produces at least three products: a bacteriocin called pesticin, lethal for nonimmune yersiniae; a protein conferring immunity to pesticin; and an outer membrane protease known as Pla (*4*). Pla is responsible for two in vitro phenotypes long associated with *Y. pestis*: weak procoagulant activity and the lysis of fibrin clots, the latter probably produced by activation of plasminogen (*5*). In this report we describe the effect of inactivation of the *pla* and *pst* (pesticin) genes on *Y. pestis* virulence.

All of our experiments were conducted with derivatives of *Y. pestis* strain KIM-10, which was initially isolated from a human plague victim in Iran in the early 1960s (*6*). The 9.5-kb plasmid in this strain has been designated pPCP1 (*4*). We constructed a series of isogenic strains containing pPCP1 mutants or cloned fragments and determined their virulence by measuring $LD_{50}$ values in subcutaneously inoculated mice (Fig. 1). All strains in which the *pla* gene was missing or inactivated (including the strain KIM1008, which carries an internal in-frame *pla* deletion) yielded $LD_{50}$ values close to $10^7$. In contrast, $LD_{50}$ values for those strains with an active *pla* gene were all less than 50. Because the latter group includes KIM1006, which contains *pla* but no other gene of pPCP1, we can conclude that *pla* is both necessary and sufficient to explain the contribution of pPCP1 to virulence. Inactivation of *pst* had no effect in these experiments.

We determined the dose capable of infecting 50% of the mice (the $ID_{50}$) by scoring both mice that died and mice that developed significant antibody titers against the *Y. pestis* capsular protein (F1 antigen) as infected. All of the strains, whether *pla*$^+$ or *pla*$^-$, were infectious at very low doses ($ID_{50}$ < 10) (Fig. 1). Many of the mice infected with *pla*$^-$ mutants had lesions at the injection site that persisted until termination of the experiment after 21 days, another indication of established infection. Histological examination of injection sites at 3 and 4 days after infection (Fig. 2) revealed mac-

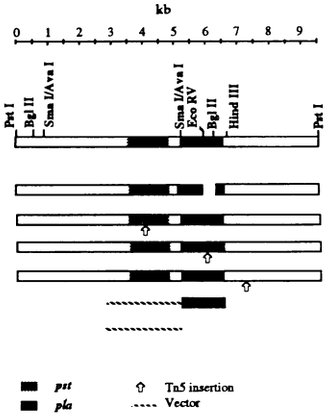
O. A. Sodeinde, Y. V. B. K. Subrahmanyam, K. Stark, Y. Bao, J. D. Goguen, Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, MA 01655.
T. Quan, Division of Vector-Borne Diseases, Centers for Disease Control, Fort Collins, CO 80522.

*Present address: Plant Science Center, Cornell University, Ithaca, NY 14853.
†Present address: Department of Neurology, Tufts University School of Medicine, Boston, MA 02111.
‡Present address: Department of Microbiology, Fujian Medical School, Fuzhou, Peoples Republic of China.
§To whom correspondence should be addressed.

roscopic masses of bacteria, as large as 1 mm, containing many more organisms than the original inoculum for both *pla*$^+$ and *pla*$^-$ strains. The one obvious difference between lesions caused by the two strains was in the number and distribution of inflammatory cells present. Lesions produced by the *pla*$^+$ strain contained few inflammatory cells. Lesions produced by the *pla*$^-$ strain contained large numbers of such cells, predominantly intact polymorphonuclear neutrophils (PMN), both in close contact with the bacteria and in a dense zone at the margin of the edematous tissue.

Consistent with published descriptions of plague pathology, large numbers of bacteria were visible in sections of liver and spleen taken from mice infected with *pla*$^+$ strains. However, no bacteria were visible in similar sections from mice infected with *pla*$^-$ strains. We confirmed this result by plating spleen and liver homogenates from mice infected subcutaneously with 1000 bacteria. All six mice inoculated with KIM1001 and examined between days 3 and 6 after infection had titers of *Y. pestis* in spleen and liver exceeding $2 \times 10^6$ bacteria per gram, whereas only two of six mice inoculated with KIM1008 had positive spleen cultures and had comparatively low titers ($2.9 \times 10^4$ and $9.0 \times 10^3$); none of the mice inoculated with KIM1008 had positive liver cultures (detection limit $1.5 \times 10^1$).

The results described above establish that *pla*$^-$ mutants delivered by subcutaneous injection produce a local infection, have greatly reduced ability to establish infection in liver and spleen, and are essentially avirulent by this route. Nonetheless, they remain highly virulent if delivered intravenously. When a group of five mice received an intravenous (i.v.) dose of 1000 bacteria of strain KIM1008, three died and the remaining pair was killed when death appeared imminent (after 5 days). Both livers and spleens of the killed animals contained large numbers of bacteria (mean titers: liver, $4.2 \times 10^6$ bacteria per gram; spleen, $1.3 \times 10^7$ bacteria per gram).




| Strain | Plasmid | Pst | Relative Pla activity | $LD_{50}$ | $ID_{50}$ | Antibody titer |
|---|---|---|---|---|---|---|
| KIM1001 | pPCP1 | + | 1.0 | $4.2 \times 10^1$ | <7.5 | 512 |
| KIM1002 | None | - | 0 | $8.8 \times 10^6$ | <3.9 | 1287 |
| KIM1008 | pPCP1(*pla*Δ10) | + | 0 | $6.0 \times 10^7$ | <6.0 | 407 |
| KIM1003 | pPCP1::Tn5(4.1) | - | 1.2 | $3.3 \times 10^1$ | <2.4 | 2048 |
| KIM1004 | pPCP1::Tn5(6.0) | + | 0 | $4.5 \times 10^6$ | <1.0 | 927 |
| KIM1005 | pPCP1::Tn5(7.2) | + | 1.3 | $3.0 \times 10^0$ | <1.0 | 512 |
| KIM1006 | pPLA7 | - | 2.5 | $2.7 \times 10^1$ | <3.1 | 256 |
| KIM1007 | pBR322Δ1 | - | 0 | $1.1 \times 10^7$ | <2.7 | 128 |

**Fig. 1.** Virulence of *Y. pestis* strains carrying pPCP1 and derivative plasmids. The strains used in this experiment are genetically identical to wild-type KIM1001 except for modification, elimination, or replacement of pPCP1 as illustrated. The gap in the *pla* gene of KIM1008 represents an internal in-frame deletion that results in synthesis of a shortened and inactive Pla protein. Numbers in parentheses after plasmid designations indicate sites of Tn5 insertion on the pPCP1 map. [For a more detailed map of pPCP1, see (*11*)]. Pst indicates the pesticin phenotype of the strains. Pla activity was measured in plasminogen activation assays. The vector used in construction of plasmid pPLA7, pBR322Δ1, is a derivative of pBR322 from which the tetracycline resistance gene has been deleted. For $LD_{50}$ and $ID_{50}$ determinations, seven groups of eight female outbred mice, 6 to 8 weeks old, were injected subcutaneously with a 0.1-ml inoculum containing from $10^1$ to $10^7$ bacteria (each group represented a tenfold difference in dose). An eighth group received a saline injection and served as an uninfected control. The inoculum consisted of bacteria grown at 26°C in heart infusion broth (Difco), washed, and resuspended in phosphate-buffered saline at the appropriate density. Mice were observed for 21 days after infection. Median lethal doses were calculated by the method of Reed and Muench (*19*). The same method was used for calculation of the $ID_{50}$ except that both survivors with significant antibody titers to the *Y. pestis* F1 capsular antigen and dead mice were scored positively. Antibody titer is the calculated geometric mean titer at the $ID_{50}$.

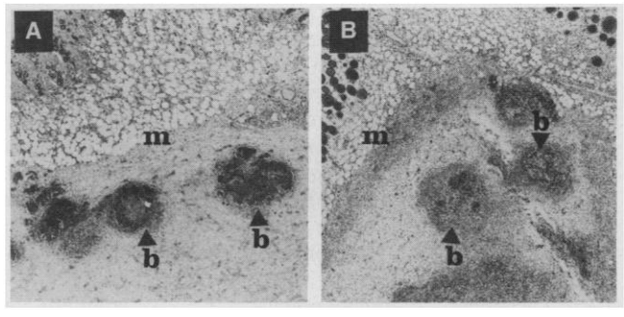



**Fig. 2.** Histopathology of subcutaneous lesions at day 3 after infection. In comparison with the virulent KIM1001 (**A**), KIM1008 (**B**) produced a lesion containing many more inflammatory cells. These are visible as a dense zone at the margin (m) of the edematous tissue, surrounding and obscuring the bacterial masses (b) and more diffusely throughout the lesion. Stain, hematoxylin-eosin; magnification: ×20.

It has previously been shown that the fibrinolytic activity of *Y. pestis* is eliminated if the fibrin film substrate is preheated to denature contaminating plasminogen (*5*), suggesting that the fibrinolysis is due to plasminogen activation. To determine if Pla does have plasminogen activator activity, we investigated its effect on plasminogen. Because the activity of Pla is rapidly lost after extraction from bacterial outer membranes, purified outer membranes from an *Escherichia coli* strain engineered to overproduce Pla were used as the source of the enzyme in these experiments. Membranes from an isogenic strain lacking Pla were included as negative controls in all assays and were always found to have no detectable activity. Human urokinase, the major cell surface–associated plasminogen activator, was used for comparison. Both Pla and urokinase cleaved human Glu-plasminogen (the major form of the zymogen in plasma) to produce products that were indistinguishable by electrophoresis on denaturing gels (*7*). Moreover, we found that the $NH_2$-terminus of the plasmin light chain produced by Pla was identical to that of the light chain produced by urokinase (Val-Val-Gly-Gly-Cys. . .), indicating that cleavage by both activators occurred at the same site. Plasmin produced by Pla was active, cleaving the chromogenic plasmin substrate Val-Leu-Lys-*p*-nitroanalide (S2251) (*8*). Pla itself had negligible activity against this substrate. Pla also lacked activity against Glu-Gly-Arg-*p*-nitroanalide (S2444) (*9*), a chromogenic substrate for urokinase.

Using S2251 to quantitate plasmin, we determined the apparent Michaelis-Menten constant ($K_m$) of membrane-bound Pla for human Glu-plasminogen (Fig. 3). Reactions conducted with whole *Y. pestis* cells yielded a $K_m$ (145 nM) close to that obtained with purified outer membranes. Surprisingly, these values are much lower than those reported for the human activators urokinase (*10*) and tissue plasminogen activator (*11*), indicating a very high affinity for this substrate. Using densitometry of Coomassie-stained gels to determine the amount of Pla in the outer membrane preparations, one can also estimate the catalytic constant

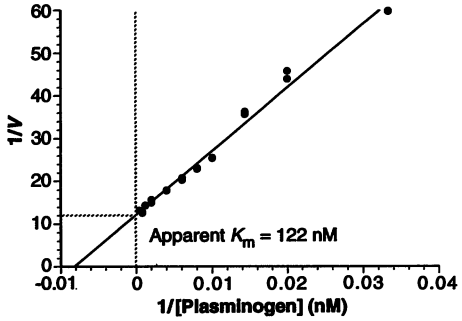



**Fig. 3.** Lineweaver-Burke plot for plasminogen activation by Pla. Reactions were conducted at 37°C and contained 300 pM Pla [as purified outer membranes from the *E. coli* Pla overproducer BL21(DE3, pPLA100)], 0.5 mM S2251, and human Glu-plasminogen at the indicated concentrations in 0.1 M tris[hydroxymethyl]aminomethane, 0.05 M 2[*N*-morpholino]ethanesulfonic acid, and 0.1% Tween 80 adjusted to pH 7.4 with acetic acid. *V* is expressed as moles of plasmin formed per mole of Pla per minute, and was calculated from the rate of *p*-nitroanalide formation by means of kinetic parameters for the hydrolysis of S2251 determined under the reaction conditions described above.

($k_{cat}$) of Pla for human Glu-plasminogen. The value obtained, 0.21 $min^{-1}$, is very low as compared with that obtained from human urokinase [89 $min^{-1}$ (*10*)]. This estimate of Pla $k_{cat}$ is probably low by about a factor of 2 because some fraction of the protease is contained within inverted membrane vesicles and does not have access to plasminogen during the activation reaction.

To determine whether Pla can activate plasminogen under more physiological conditions, we conducted clot lysis time experiments in reaction mixtures containing high concentrations (33%) of human and rat plasma (*12*). Pla was active under these conditions and had about 1% (human plasma) to 3% (rat plasma) of the activity of human urokinase on a molar basis.

On the basis of the activity of whole-cell suspensions and the $k_{cat}$ value given above, we estimate that a *Y. pestis* cell contains between 10,000 and 20,000 Pla molecules. In comparison, a human cell line producing high amounts of urokinase receptor has the capacity to bind about 50,000 molecules of urokinase (*13*). (In vivo, active urokinase is probably found only in the context of such receptors.) Assuming that the receptors were saturated with active urokinase, and using a conservative estimate of cell surface area (the surface area of a sphere 20 mm in diameter) and of the activity of Pla relative to that of urokinase (1%), we find that the activities per unit surface of these cells and of *Y. pestis* are essentially equal. Thus, the plasminogen activator activity of *Y. pestis* is apparently high enough to be physiologically significant during infection despite the low $k_{cat}$ of Pla.

We have previously reported that Pla is responsible for the procoagulant activity of *Y. pestis* (*4*). Because this activity is weak—high concentrations of bacteria ($>10^8$ per ml) and incubation for several hours are both required for positive results in plasma clotting assays—it seems unlikely to play an important role during infection of mammalian hosts. This activity has been demonstrated only in the rabbit plasma clotting assay of Beesley *et al.* (*5*). Using this assay, but substituting plasma from other species and adding appropriate inhibitors, we have obtained additional data supporting this hypothesis. Most importantly, we find that procoagulant activity cannot be demonstrated in human, mouse, or rat plasma at either 26° or 37°C, even when *tans*-4-aminomethylcyclohexane-1-carboxylic acid (AMCA) (5 mM) is added to block plasmin-mediated fibrinolysis. Nonetheless, *Y. pestis* is highly virulent in all of these species. Moreover, clot formation in rabbit plasma is not affected by the thrombin inhibitor Nα-(2-naphthalenesulfonylglycyl)-4-amidino-DL-phenylalaninepiperidide (α-NAPAP) (1 mM), indicating that Pla is not a true (thrombin-activating) coagulase. On the basis of these results, the most likely mode of action for Pla in clot formation is proteolysis of rabbit fibrinogen—at a sequence (or sequences) not found in fibrinogens from the other species tested—to produce fibrin-like products. Consistent with this mechanism, "fibrin" in the clots formed by Pla does not contain the covalent cross-links between fibrin monomers characteristic of normal clots (*14*).

Given the plasminogen activator activity of Pla, it may seem paradoxical that clots can be formed at all in Pla-containing plasma. However, active plasmin in plasma is rapidly inactivated by both $\alpha_2$-antiplasmin and $\alpha_2$-macroglobulin if not bound to fibrin, and it is likely that plasmin activated by Pla is exhausted before the slowly forming clot appears in rabbit plasma experiments.

The low number of inflammatory cells at injection sites containing *pla*$^+$ bacteria suggests that Pla reduces production of chemoattractant activity. Before the development of an adaptive immune response, the major chemoattractant at a site of Gram-negative infection is probably the C5a peptide released from complement C5 during complement activation by the alternative pathway. Thus, Pla may interfere with steps in the complement activation pathway before release of C5a or by degrading C5a directly. Production of C5 convertase is a complex process involving several proteins [C3b (or C3), factor B, factor D, and properdin] and is therefore vulnerable to protease attack. Moreover, assembly of both C5 convertase and its precursor, C3 convertase, occurs on the bacterial surface, where Pla is in high concentration. Our initial investigation of the interaction of Pla with the complement system indicates that Pla cleaves C3, but not factor B (*15*). We find that Pla mutants are completely resistant to high concentrations (90%) of human serum, which is in agreement with the ability of Pla mutants to produce a sustained infection in mice. Thus, although interaction of Pla with the complement system may be important, it is clear that the major function of Pla is not that of providing resistance to complement-mediated lysis.

The data presented above establish that the Pla protease is an important virulence factor in *Y. pestis*. The observation that Pla mutants are highly infectious and proliferate at injection sites after subcutaneous inoculation, but—unlike the wild-type parent—fail to accumulate in liver and spleen, suggests that the major function of the Pla protease is in dissemination of the bacteria from infection sites. This conclusion is supported by the observation that a Pla mutant can, in effect, be complemented when the bacteria are injected intravenously: under this regime the bacteria are lethal for mice at low doses and accumulate in liver and spleen to the same extent as the wild-type parent.

Our results show that neither pesticin activity nor the procoagulant activity of Pla is important for virulence of *Y. pestis* for mice and that Pla does not play a primary role in resistance to innate bacteriostatic or bactericidal host defenses. Instead, Pla appears to act by breaching barriers to the spread of infection. There are several potential mechanisms by which plasminogen activation could have this effect. (i) Fibrin deposition is a common response to bacterial infection, and it has been shown that fibrin deposited around bacteria can effectively trap them (*16*). This mechanical restraint might be abolished by plasmin-mediated fibrinolysis. (ii) Contacts between inflammatory cells and fibrin may play an important role in abscess morphogenesis, and excess plasmin activity may result in a poorly organized and ineffective structure. (iii) In tumor metastasis, plasmin is thought to degrade extracellular and basement membrane proteins both directly and by the activation of procoagulases, thus promoting cell migration by the elimination of mechanical restraints (*17*). Damage to these barriers may also interfere with confinement of bacteria.

An alternative hypothesis is that interference with the amplification step of alternative pathway complement activation inhibits formation of an effective bacteria-confining abscess by reducing chemoattraction of PMN to the infection site. This hypothesis correlates more directly with the in vivo observation of reduced PMN accu-

mulation and also predicts the surface location of Pla. The plasminogen activation and chemoattractant reduction hypotheses are not mutually exclusive. These two mechanisms may be synergistic by affecting both cellular and noncellular processes contributing to containment of the bacteria. Direct in vivo tests of hypotheses regarding Pla activity will be essential to understand its mechanism of action.

Whatever the mechanism of Pla action, our findings may have broad implications for other Gram-negative infections. Homologs of *pla* have been found in *Escherichia coli* (*ompT*, 50% homology) and *Salmonella typhimurium* (*prtA*, 70% homology) (*14*). We have recently sequenced another in *Salmonella typhi* that is very similar to the *S. typhimurium* gene. In all of these organisms, the *pla* homologs are chromosomal and have no known physiological function. The role of Pla in plague implies that this class of proteases may contribute, in a more subtle way, to the capacity of less virulent species to produce disseminated infection. Such infections are a major source of mortality for immunocompromised patients. As we have noted (*14*), the homologies described above also suggest that *Y. pestis* acquired *pla* from a member of the *Salmonella* lineage. Given the importance of this gene to the life cycle of *Y. pestis* in nature, acquisition of the pPCP1 plasmid was clearly a major event in the evolution of this pathogen from *Y. pseudotuberculosis*. This argues against the notion that *Y. pestis* has arisen repeatedly from *Y. pseudotuberculosis* by acquisition of gene-inactivating mutations (*18*). It also presents another example of the importance of mobile genetic elements in the evolution of bacterial virulence.

## REFERENCES AND NOTES


1. H. Bercouvier and H. H. Mollaret, in *Bergeys Manual of Systematic Bacteriology*, N. R. Kreig and J. G. Holt, Eds. (Williams and Wilkins, Baltimore, 1984), vol. 1, pp. 498–509.
2. R. R. Brubaker, E. D. Beesley, M. J. Surgalla, *Science* **149**, 422 (1965).
3. T. Butler, *Plague and Other* Yersinia *Infections* (Plenum, New York, 1983), pp. 118–140.
4. O. A. Sodeinde and J. D. Goguen, *Infect. Immun.* **56**, 2743 (1988).
5. E. D. Beesley, R. R. Brubaker, W. A. Jansen, M. J. Surgalla, *J. Bacteriol.* **165**, 19 (1967).
6. M. J. Finegold, J. J. Petery, R. F. Berendt, H. V. Adams, *Am. J. Pathol.* **53**, 99 (1961).
7. Human Glu-plasminogen (10 μM) was incubated with purified Pla-containing *E. coli* outer membranes (~2 μM Pla) in 0.1 M tris-HCl buffer (pH 7.5) for 20 min at 37°C. Bacterial membranes were removed by centrifugation, and a sample of the supernatant was separated on an SDS-polyacrylamide gel. A sample of the same plasminogen activated with urokinase was run in an adjacent lane for comparison and was indistinguishable from the Pla-treated sample. A larger amount of the supernatant was separated on a preparative gel and transferred to Immobilon-P (Millipore). The band corresponding to the light chain of urokinase-activated plasmin was excised and the $NH_2$-terminal sequence determined.
8. J. H. Verheijen, E. Mullart, G. T. G. Chang, G. Wijngaards, *Thromb. Haemostasis* **48**, 266 (1982).
9. R. Lottenberg, U. Christensen, C. M. Jackson, P. L. Coleman, *Methods Enzymol.* **80**, 341 (1981).
10. S. W. Peltz, T. A. Hardt, W. F. Mangel, *Biochemistry* **21**, 2798 (1982).
11. M. Hoylaerts, D. C. Rijken, H. R. Lijnen, D. Collen, *J. Biol. Chem.* **257**, 2912 (1982).
12. Seventy-five microliters of fresh human or rat plasma were added to 150 μl of 0.1 M phosphate buffer (pH 7.2) containing 2 units of human thrombin and a range of concentrations of either Pla [as purified outer membranes from the *E. coli* Pla over-producer BL21(DE3, pPLA100)] or human urokinase. Clots formed immediately, and the time required for complete lysis was observed. The logarithm of lysis time was plotted against the logarithm of enzyme concentration. Linear regression lines fitted to these plots were essentially parallel, and the mean distance between them was used as a measure of relative activity. Membranes from the *pla*$^-$ control strain BL21(DE3, pBS KS$^-$) had no detectable activity.
13. P. M. Stoppelli *et al.*, *Cell* **45**, 675 (1986).
14. O. A. Sodeinde and J. D. Goguen, *Infect. Immun.* **57**, 1517 (1989).
15. Five micrograms of factor B or C3 were incubated alone, with *pla*$^-$ *E. coli* outer membranes (400 ng of total protein), or with *pla*$^+$ outer membranes (400 ng of total protein, 200 ng of Pla). The reaction mixtures were then analyzed by electrophoretic separation on denaturing gels. Factor B was unaffected by any of these treatments, whereas the α chain of C3 had higher mobility after treatment with *pla*$^+$ membranes, indicating removal of a fragment of about 10 kD.
16. D. L. Dunn and R. L. Simmons, *Surgery* **92**, 513 (1982).
17. P. Mignatti, E. Robbins, D. B. Rifkin, *Cell* **47**, 487 (1986).
18. R. Rosqvist, M. Skurnik, H. Wolf-Watz, *Nature* **334**, 522 (1988).
19. L. J. Reed and H. Muench, *Am. J. Hyg.* **27**, 493 (1938).


20. Supported by grant AI22176 from the National Institutes of Health (J.G.). We thank I. Joris, G. Mano, and D. Tipper for discussions, M. Apple for histological preparations, and Yi Bao for general technical assistance.

1 June 1992; accepted 28 July 1992


# Activity-Dependent Decrease in NMDA Receptor Responses During Development of the Visual Cortex


Giorgio Carmignoto* and Stefano Vicini



**Plasticity of the developing visual system has been regarded as the best model for changes of neuronal connections under the influence of the environment. *N*-methyl-D-aspartate (NMDA) receptors are crucial for experience-dependent synaptic modifications that occur in the developing visual cortex. NMDA-mediated excitatory postsynaptic currents (EPSCs) in layer IV neurons of the visual cortex lasted longer in young rats than in adult rats, and the duration of the EPSCs became progressively shorter, in parallel with the developmental reduction in synaptic plasticity. This decrease in NMDA receptor–mediated EPSC duration is delayed when the animals are reared in the dark, a condition that prolongs developmental plasticity, and is prevented by treatment with tetrodotoxin, a procedure that inhibits neural activity. Application of L-glutamate to outside-out patches excised from layer IV neurons of young, but not of adult, rats activated prolonged bursts of NMDA channel openings. A modification of the NMDA receptor gating properties may therefore account for the age-dependent decline of visual cortical plasticity.**


In the visual cortex, activation of the NMDA subtype of glutamate receptor contributes to normal excitatory synaptic transmission (*1*) and is required for the experience-dependent modifications of synaptic connections that occur during a restricted period of early postnatal development in kittens (*2*). After the animal reaches adulthood, the cortical synaptic organization can no longer be modified by visual experience (*2*). To test whether this reduction in plasticity is associated with a modification of NMDA receptor responses, we studied functional properties of the NMDA receptor in visual cortical neurons of the rat during development, from the time before eye opening to adulthood. We used whole cell and excised patch current recordings in brain slices (*3–5*) from 163 neurons located in layer IV out of a total of 198 visual cortical neurons (*6*).

We studied NMDA responses by analyzing the EPSC evoked by stimulation of the borders between the white matter and layer VI (*7*). In all neurons with voltages clamped at −70 mV holding potential, the EPSC was composed of a fast-decaying component (*8*), which was abolished by the non-NMDA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*F*)quinoxaline (NBQX) (10 μM), and a slower tail. This latter component was mediated by NMDA receptors because it was abolished by the selective NMDA antagonist 3-[(±)-2-2-carboxypiperazin-4-yl]-propyl-1-phosphonic


FIDIA Georgetown Institute for the Neurosciences, Georgetown University School of Medicine, 3900 Reservoir Road, NW, Washington, DC 20007.

*To whom correspondence should be addressed.