VIII preparation cannot be ruled out.

The inhibition of thrombin-activated human factor VIII by diisopropyl phosphorofluoridate has been difficult to demonstrate. This is due in part to the rapid inactivation of the activated molecule even in the presence of high concentrations of $CaCl_2$. Similar results have also been observed by Switzer and McKee (*12*). Thus, the human preparation differs markedly from the bovine preparation in its stability after activation by thrombin.

GORDON A. VEHAR
EARL W. DAVIE
Department of Biochemistry, University of Washington, Seattle 98195

References and Notes

1. The nomenclature for the various coagulation factors is that recommended by an international nomenclature committee [I. Wright, *J. Am. Med. Assoc.* **170**, 325 (1959)].
2. E. W. Davie and K. Fujikawa, *Annu. Rev. Biochem.* **44**, 799 (1975).
3. R. L. Lundblad and E. W. Davie, *Biochemistry* **3**, 1720 (1964).
4. R. G. Macfarlane, R. Biggs, B. J. Ash, K. W. E. Denson, *Br. J. Haematol.* **10**, 530 (1964).
5. C. Hougie, K. W. E. Denson, R. Biggs, *Thromb. Diath. Haemorrh.* **18**, 211 (1967); H. C. Hemker and M. J. P. Kohn, *Nature (London)* **215**, 1201 (1967); B. Østerud and S. I. Rapaport, *Biochemistry* **9**, 1854 (1970); T. F. Chuang, R. B. Sargeant, C. Hougie, *Biochim. Biophys. Acta* **273**, 287 (1972).
6. K. Fujikawa, M. H. Coan, M. E. Legaz, E. W. Davie, *Biochemistry* **13**, 5290 (1974).
7. K. Fujikawa, K. Titani, E. W. Davie, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3359 (1975).
8. K. Fujikawa, M. E. Legaz, H. Kato, E. W. Davie, *Biochemistry* **13**, 4508 (1974); D. L. Enfield, L. H. Ericsson, K. Fujikawa, K. Titani, K. A. Walsh, H. Neurath, *FEBS Lett.* **47**, 132 (1974); K. Kurachi, K. Fujikawa, G. Schmer, E. W. Davie, *Biochemistry* **15**, 373 (1976).
9. S. I. Rapaport, S. Schiffman, M. J. Patch, S. B. Ames, *Blood* **21**, 221 (1963); R. Biggs, R. G. Macfarlane, K. W. E. Denson, B. J. Ash, *Br. J. Haematol.* **11**, 276 (1965); P. A. McKee, J. C. Anderson, M. E. Switzer, *Ann. N.Y. Acad. Sci.* **240**, 8 (1975); M. E. Legaz, M. J. Weinstein, C. M. Heldebrant, E. W. Davie, *ibid.*, p. 43.
10. W. G. Owen and R. H. Wagner, *Thromb. Diath. Haemorrh.* **27**, 502 (1972).
11. H. A. Cooper, F. F. Reisner, M. Hall, R. H. Wagner, *J. Clin. Invest.* **56**, 751 (1975). A difference in the gel filtration pattern of bovine factor VIII before and after thrombin treatment has also been observed by Switzer and McKee (*12*).
12. M. E. Switzer and P. A. McKee, personal communication.
13. J. E. Brown, R. F. Baugh, R. B. Sargeant, C. Hougie, *Proc. Soc. Exp. Biol. Med.* **147**, 608 (1974); T. R. Griggs, H. A. Cooper, W. P. Webster, R. H. Wagner, K. M. Brinkhous, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2814 (1973); T. R. Griggs, W. P. Webster, H. A. Cooper, R. H. Wagner, K. M. Brinkhous, *ibid.* **71**, 2087 (1974).
14. D. E. G. Austen, *Br. J. Haematol.* **27**, 89 (1974).
15. H. J. Weiss, L. L. Phillips, W. Rosner, *Thromb. Diath. Haemorrh.* **27**, 212 (1972); M. E. Rick and L. W. Hoyer, *Blood* **42**, 737 (1973).
16. M. E. Switzer and P. A. McKee, *J. Clin. Invest.* **57**, 925 (1976).
17. M. E. Rick and L. W. Hoyer, *Nature (London)* **252**, 404 (1974); *Thromb. Res.* **7**, 909 (1975).
18. E. A. Beck, P. Bachmann, P. Barbier, M. Furlan, *Thrombos. Haemostas. (Stuttgart)* **35**, 186 (1976).
19. M. C. Poon and O. D. Ratnoff, *Blood* **48**, 87 (1976).
20. M. E. Legaz and E. W. Davie, *Methods Enzymol.* **45**, 83 (1976).
21. A. R. Thompson and E. W. Davie, *Biochim. Biophys. Acta* **250**, 210 (1971).
22. K. Kurachi, G. Schmer, M. A. Hermodson, D. C. Teller, E. W. Davie, *Biochemistry* **15**, 368 (1976).
23. H. A. Cooper, T. R. Griggs, R. H. Wagner, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2326 (1973).
24. Supported in part by NIH grant HL 16919-02. We thank M. E. Switzer and P. A. McKee for helpful information and K. Fujikawa and W. Kisiel for valuable discussions.

28 September 1976; revised 29 March 1977

Antigenic Shift of Visna Virus in Persistently Infected Sheep

Abstract. *Visna viruses isolated from persistently infected sheep were antigenically distinct from the plaque-purified virus used for inoculation. The selection of antigenic variants under antibody pressure, thought to occur in vivo, was reproduced in sheep cell cultures inoculated with plaque-purified visna virus and maintained in antibody. Antigenic shift may be a mechanism for persistence of virus in slow or recurrent viral infections.*

Visna of sheep is one of the slow virus infections characterized by a prolonged incubation period and protracted disease (*1*). Unlike the etiologic agent of scrapie, the other prototype slow infection, the visna agent is a conventional virus that elicits an immune response in sheep and causes an inflammatory, demyelinating disease of the brain and spinal cord (*2*). Although visna virus is cytolytic for sheep cells in culture, the virus undergoes minimal replication in vivo (*3*).

The restriction of replication in sheep occurs immediately after inoculation, but virus can be isolated from many tissues—including peripheral blood leukocytes (PBL), by tissue explantation or cocultivation—for months to years after inoculation (*4, 5*). Furthermore, ease of virus recovery from PBL varies among individual animals but is not affected by the virus-neutralizing antibody which develops later in the infection (*6*). Regardless of the duration of the infection, tissues from infected sheep only occasionally yield cell-free virus, virus antigens are rarely found by immunofluorescence, and virus particles are not seen by electron microscopy (*4, 7*). These phenomena have been explained by the demonstration that visna virus, a retravirus containing an RNA-directed DNA polymerase, exists in vivo primarily as provirus (*8*). In contrast to some tumor-associated retraviruses, the proviral DNA does not hybridize with normal sheep DNA (*9*). In infected sheep, sequestration of the genome of visna virus as provirus may explain virus persistence in the immune animal; however, other mechanisms must be sought to explain the development of disease months to years after infection. The unpredictable development of disease might be explained by the intermittent release of antigenically altered virus which could escape neutralization in the immune host (*5*). To test this hypothesis, viruses isolated from sheep inoculated with visna virus were compared to the parent virus for possible antigenic shift.

Four Hampshire outbred lambs were inoculated intracerebrally with approximately 10^6 TCD_{50} (50 percent tissue culture infectious doses) of strain 1514 of visna virus which had been plaque-purified. Blood was taken from the sheep at regular intervals after inoculation, and virus isolations were accomplished by cocultivation of 10^5 to 10^6 PBL on monolayer cultures of indicator sheep choroid plexus cells (*4*). Peripheral blood leukocytes from uninoculated control sheep failed to yield virus.

All four inoculated sheep responded with neutralizing antibody to the inoculum virus within 6 months, but serums failed to neutralize another prototype strain of visna virus, D1-2 (Fig. 1) (*10*). Both virus strains, 1514 and D1-2 were derived from a common ancestral strain K796 (*4*). Four strains of virus isolated from leukocytes of the four sheep were examined by neutralization with isologous and homologous serums. Each leukocyte-derived virus was found to be distinct from the parent strain. Viruses isolated from leukocytes of sheep Nos. 1, 2, and 3 could not be neutralized by immune serums from the three animals but were neutralized by low dilutions of serum from sheep No. 4. The virus recovered from leukocytes of the fourth sheep was neutralized by isologous serum, but this serum neutralized the inoculum virus at higher dilutions (Fig. 1).

The viruses from leukocytes were propagated in vitro for three or more passages at limiting dilutions, and neutralization tests indicated that they had maintained their antigenic identity (*6*). These agents were identified as visna viruses by (i) neutralization by serum from a goat hyperimmunized with both prototype viruses, 1514 and D1-2, and (ii) fluorescent antibody staining with hyperimmune goat serum on cell cultures infected with each of the viruses (*6*). In contrast to postinfection immune sheep serums which tended to have narrow neutralizing activity, hyperimmunization of the goat induced neutralizing antibodies whose activity apparently extended beyond the antigenic determinants of the two immunogens.

Complement fixation (CF) tests, with

the two prototype viruses as antigens, failed to distinguish clearly between visna viruses (Table 1). In a study of seven sheep (including the four shown in Fig. 1) that were infected with three strains of virus, serums from only three animals (Nos. 3, 5, and 6) had significant differences in levels of CF antibody between the inoculum and heterologous viruses. In contrast, serums from all seven sheep had monospecific neutralizing antibody that was directed to the virus in the inoculum. These results suggested that visna viruses share CF antigens but have unique proteins which can be differentiated in neutralization tests. A similar observation has been made with serums from Icelandic sheep (*11*). Since our sheep were inoculated with plaque-purified virus, and proviral DNA was demonstrated in tissue, we interpreted the derivation of the antigenically distinct leukocyte viruses as mutants originating from viral DNA templates in tissue cells. These mutants could then replicate in the presence of neutralizing antibody that was directed exclusively to the parental virus in the inoculum. To test this hypothesis in vitro, strain 1514 of visna virus was plaque-purified twice and inoculated at low multiplicity onto cell cultures. After 1 hour of incubation at 37°C, the inoculum was removed; and the cultures were overlaid with medium containing immune serum (diluted 1 : 50) from sheep No. 4. After the virus cytopathic effect was maximal, a portion of the supernatant was reacted with the same dilution of antibody at 37°C for 30 minutes and reinoculated onto cell cultures. These cultures were then maintained in antibody for a prolonged period until cytopathic effects developed. Viruses obtained after five passages in antibody were then cultivated in antibody-free medium and compared to the original parent virus in neutralization tests (Table 2). Three viruses derived in vitro showed significant antigenic differences from the parental strain, and neutralization with serum from sheep No. 1 and the hyperimmune goat suggested that these viruses also differed from one another.

Thus, although visna viruses appear antigenically stable in cell cultures, infections of sheep which develop antibody, or infection of cell cultures treated with antibody, give rise to selected populations of virus with sufficient antigenic alteration to escape neutralization by antibodies directed against parental determinants. The lack of neutralizing antibody in sheep Nos. 1, 2, and 3 to their respective leukocyte viruses may indicate that the latter were recent mutants, that they had undergone insufficient replication to evoke an antibody response, or that antibody formation in the animals continued to be directed toward the determinants of the inoculum virus, so-called "original antigenic sin" (*12*). Further studies in other sheep have shown that viruses isolated from PBL do not shift antigenically from parental strains prior to development of neutralizing antibody in the animal. The observation that leukocyte-derived viruses could not be differentiated antigenically from parent viruses in CF tests suggests that the antigenic shift probably involves a specific envelope glycoprotein recognized by neutralizing antibodies.

Antigenic shift within the individual

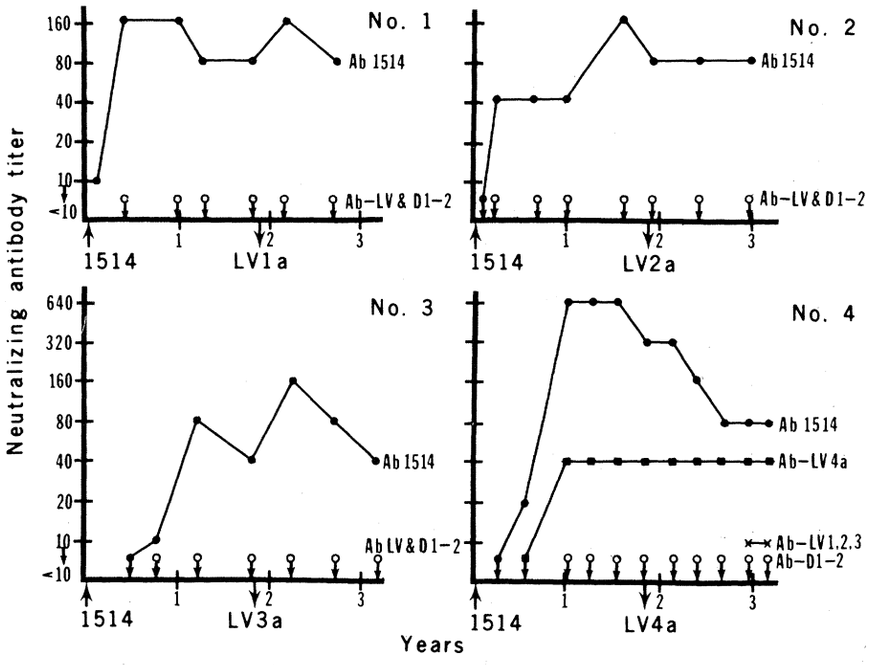

Fig. 1. Immune response and virus isolations from four sheep inoculated intracerebrally with plaque-purified visna virus strain 1514. Neutralizing antibody (*Neut. Ab.*) titers are reciprocals of 50 percent end-point dilutions of serums which neutralized 100 TCD_{50} of virus. The upper plot (•) indicates levels of antibody (*Ab*) against the inoculum virus; (♀) failure of serums at 1 : 10 dilutions to neutralize virus; *LV* refers to the four lymphocyte-derived viruses isolated at the time indicated (↓) from each of the four sheep; (■) levels of antibody in serum of sheep No. 4 to the lymphocyte virus derived from the sheep No. 4; (x) low level of antibody in serum of sheep No. 4 to lymphocyte viruses derived from sheep Nos. 1, 2, and 3. None of the sheep produced antibody to virus strain D1-2, the other prototype visna virus.

Table 1. Comparison of serologic responses of sheep to different strains of visna viruses. The SN titers are reciprocals of 50 percent end-point dilutions of serums which neutralized 100 TCD_{50} of each virus. The CF titers are reciprocals of 50 percent end-point dilution of the same serums, which fixed four units of guinea pig complement.

Sheep No.	Virus inoculation	Postinoculation (months)	SN titer		CF titer	
			1514	D1-2	1514	D1-2
1	1514	13	80	<10	48	96
2	1514	15	160	<10	6	<6
3	1514	18	40	<10	96	<6
4	1514	13	640	<10	192	192
5	D1-2	3	<10	80	48	192
6	D1-2	3	<10	80	96	768
7	LV1a	4	<10	<10	192	192

Table 2. Antigenic differences among parental and visna virus mutants derived in vitro. Titers are reciprocals of 50 percent end-point dilutions of serums which neutralized 100 TCD_{50} of each virus.

Serum	Virus			
	Parental 1514	Derived in vitro		
		1	2	3
Sheep No. 4	160	20	10	10
Sheep No. 1	160	40	20	<10
Goat, hyperimmune	2560	640	80	80

host animal is well known in chronic protozoal infections (*13*). However, the only prior example of viral antigenic shift within a persistently infected host animal has been in equine infectious anemia (EIA) virus infections of horses (*14*). In this disease, recurrences of viremia are associated with hemolysis and fever. The disease remits with the development of neutralizing antibody and relapses when antigenically altered virus appears in the plasma. The EIA virus is also a retravirus (*15*), but lysogenic infection of horses has yet to be demonstrated. The chronic demyelinating disease in sheep caused by visna virus does not appear to be a remitting and relapsing disease, but the apparent progressive neurological disease may represent a cumulative effect of clinically inapparent attacks induced by intermittent release of antigenically altered virus. Our studies of visna suggest that lysogeny and antigenic shift of a virus may combine to provide a unique mechanism for remitting and relapsing disease. Such mechanisms of pathogenesis might be considered in multiple sclerosis or relapsing nonneurological diseases of suspected viral etiology.

O. NARAYAN
D. E. GRIFFIN
J. CHASE

Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

References and Notes

1. B. Sigurdsson, *Br. Vet. J.* **110**, 341 (1954b).
2. ——, P. A. Palsson, V. Bogaert, *Acta Neuropathol.* **1**, 343 (1962); B. Sigurdsson, P. A. Palsson, H. Grisson, *J. Neuropathol. Exp. Neurol.* **16**, 389 (1957).
3. H. Thormar and P. A. Palsson, *Perspect. Virol.* **5**, 291 (1967); O. Narayan, A. M. Silverstein, D. Price, R. T. Johnson, *Science* **183**, 1202 (1974).
4. O. Narayan, D. E. Griffin, A. Silverstein, *J. Infect. Dis.*, in press.
5. M. Gudnadottir, *Prog. Med. Virol.* **18**, 336 (1974).
6. O. Narayan and D. E. Griffin, in preparation.
7. G. Georgsson, P. A. Palsson, H. Panitch, N. Nathanson, G. Petursson, *Acta Neuropathol.*, in press.
8. A. T. Haase, L. Stowring, O. Narayan, D. Griffin, D. Price, *Science* **195**, 175 (1977).
9. A. T. Haase and H. E. Varmus, *Nature (London) New Biol.* **245**, 237 (1973).
10. Serum neutralization tests were performed in vitro by inoculation of sheep choroid plexus cells in microtiter plates with equal volumes of 100 TCD_{50} of virus and doubling dilutions of serums. Results were read 8 days later (*4*).
11. G. Petursson, N. Nathanson, G. Georgsson, H. Panitch, P. Palsson, *Lab. Invest.* **35**, 402 (1976).
12. T. Francis, Jr., F. M. Davenport, A. V. Hennessy, *Trans. Assoc. Am. Physicians* **66**, 231 (1953).
13. I. N. Brown, K. N. Brown, L. A. Hills, *Immunology* **14**, 127 (1968).
14. Y. Kono, K. Kobayashi, Y. Fukunaga, *Arch. Gesamte Virusforsch.* **41**, 1 (1973).
15. H. P. Charman, S. Bladen, R. Gilden, L. Coggins, *J. Virol.* **19**, 1073 (1976).
16. This work was supported by grants from the Public Health Service and by the Hamilton Roddis Foundation. D.G. is an investigator at the Howard Hughes Medical Institute.

26 November 1976; revised 7 February 1977

New Genetic Marker in Human Parotid Saliva (Pm)

Abstract. *In 195 parotid saliva samples collected at random from a Japanese population, two phenotypes were observed by electrophoresis in acid-urea starch gels. The protein showing polymorphisms was detected in the middle zone between Pa and Pb, and was tentatively designated Pm. Inheritance was controlled by a dominant allele at an autosomal locus. The frequencies of the genes determining these phenotypes were, for the Japanese population studied,* $Pm^+ = .38 \pm .03$, $Pm^- = .62 \pm .03$.

A new genetic polymorphism from parotid salivary proteins is described herein. By means of acid-urea starch gel electrophoresis, Azen (*1*, *2*) detected a polymorphism in parotid basic proteins (Pb) of American blacks and, using a similar system, Friedman *et al.* (*3*) detected a polymorphism in an acidic salivary protein (Pa) present in whole, parotid, and submandibular saliva of certain individuals. By a modification of these methods, we have detected another genetic polymorphism in a Japanese population. This polymorphic protein is tentatively designated Pm (salivary parotid middle-band protein), and separates electrophoretically in the zone between Pa and Pb.

Determination of Pm phenotypes was also possible by the method described by Azen (*1*), and we used the following conditions for accurate typing. Samples of parotid saliva (whole saliva that was immediately frozen and lyophilized) were collected in glass test tubes with a double-chamber cup of the Curby type; the samples were rapidly frozen, lyophilized, and stored at −20°C for later analysis. Just before being subjected to electrophoresis, the dried samples were dissolved in gel buffer solution containing 8*M* urea in order to concentrate the proteins by approximately ten times. This treatment facilitated separation of the Pm band. The starch gel contained: starch (Connaught, Toronto), 79 g; urea, 120 g; commercial aluminum lactate, 3.53 g; and lactic acid, 11.4 ml in 400 ml

Table 1. Distribution of Pm phenotypes among Pa(+) and Pa(−) individuals. All 145 samples were of Pb (1-1) type. Observed and expected values were compared: 2 by 2 contingency, $\chi^2 = 1.066$; $.30 < P < .50$.

Individual	Pm phenotype				
	Pm(+)	Expected value	Pm(−)	Expected value	Total
Pa(+)	31	34	27	24	58
Pa(−)	54	51	33	36	87
Total	85		60		145

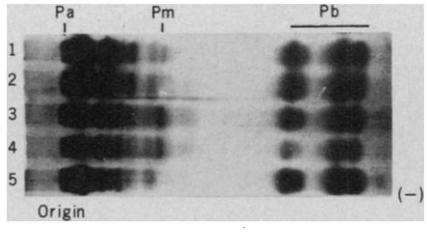

Fig. 1. Photograph of a gel on which parotid saliva was subjected to electrophoresis according to the method described by Azen (*1*). Channels 1 to 3 show the results of confirmation tests: mixtures (equal volumes) of Pm(+) and Pm(−) samples in channel 1; Pm(−) and Pm(−) samples in channel 2; and Pm(+) and Pm(+) samples in channel 3. Channel 4, Pm(+) from individual A; channel 5, Pm(−) from individual B.

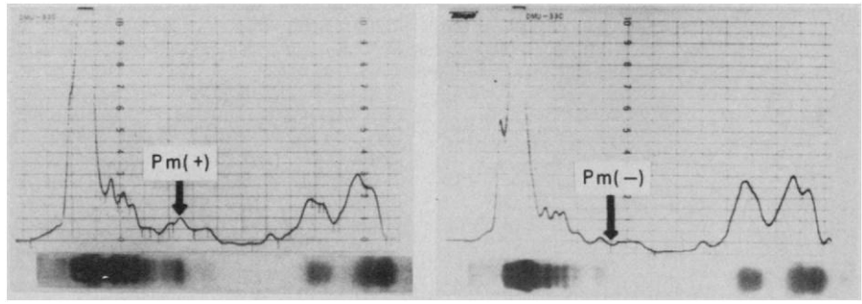

Fig. 2. Densitometric tracing patterns of Pm(+) and Pm(−) parotid saliva samples after electrophoresis. The Pm(+) sample has a peak in the zone indicated by the arrow, while the Pm(−) sample lacks this peak.