there was no survival in any of these immunized animals or control animals after inoculation with different numbers of the virulent RH strain of toxoplasma.

This is, to our knowledge, the first demonstration of immunity to intracellular bacteria induced by an intracellular protozoan. Of interest in relation to this observation are the few previously reported experimental examples of "interference" between other heterologous organisms [fungus-rickettsia (*13*), virus-bacteria (*14*), bacteria-virus (*15*), and virus-virus unrelated to interferon production (*16*)]. In these latter systems, however, the demonstration of immunity has often been dependent upon the choice of specific routes of administration of the infecting agents and upon the strict selection of intervals between primary infection with one agent and secondary challenge with the other. Despite lack of these restrictions in the toxoplasma-bacteria system and what appears to be a more lasting and perhaps more striking immunity in the experiments described here, the underlying mechanisms of resistance in all these experimental models may be similar.

Note added in proof: Since acceptance of this report, additional experiments have been performed which demonstrate that mice infected with listeria may in fact resist lethal toxoplasma challenge. The resistance observed in these experiments was elicited with a strain of toxoplasma less virulent than the RH strain previously employed in challenge studies.

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References and Notes

1. G. B. Mackaness, *J. Exp. Med.* **116**, 381 (1962).
2. ———, *ibid.* **120**, 105 (1964).
3. R. V. Blanden, G. B. Mackaness, F. M. Collins, *ibid.* **124**, 585 (1966).
4. J. S. Remington, L. Jacobs, M. L. Melton, *J. Infect. Dis.* **108**, 163 (1961).
5. G. Huldt, *Acta Path. Microbiol. Scand.* **68**, 592 (1966).
6. J. K. Frenkel, *J. Immunol.* **98**, 1309 (1967).
7. G. B. Mackaness, R. V. Blanden, F. M. Collins, *J. Exp. Med.* **124**, 573 (1966).
8. M. M. Freshman, T. C. Merigan, J. S. Remington, I. E. Brownlee, *Proc. Soc. Exp. Biol. Med.* **123**, 862 (1966).
9. W. J. Kleinschmidt, J. C. Cline, E. B. Murphy, *Proc. Nat. Acad. Sci. U.S.* **52**, 741 (1964). Statolon was supplied by Dr. W. J. Kleinschmidt, Eli Lilly and Company.
10. T. C. Merigan, *Nature* **214**, 416 (1967). Pyran was supplied by Dr. T. C. Merigan, Stanford University School of Medicine.
11. S. Baron and C. E. Buckler, *Science* **141**, 1061 (1963).
12. Levels of interferon were performed by Dr. T. C. Merigan.
13. S. B. Salvin and E. J. Bell, *J. Immunol.* **75**, 57 (1955).
14. G. Dalldorf, S. M. Cohen, J. M. Coffey, *ibid.* **56**, 295 (1947).
15. F. L. Horsfall, Jr., and M. McCarty, *J. Exp. Med.* **85**, 623 (1947).
16. R. R. Wagner and R. M. Snyder, *Nature* **196**, 393 (1962).
17. Supported by PHS grant AI-04717 and a grant from The John A. Hartford Foundation, Inc.

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Recombination of Influenza A Viruses of Human and Animal Origin

Abstract. *Simultaneous infection of the allantoic sac of the chick embryo with influenza A/equine 1/56 and any of three recombinants derived from human influenza viruses produced stable hybrids with antigens from each parent strain. These hybrids contain the hemagglutinin protein of the equine virus and the neuraminidase of the human strains. The experiments demonstrate genetic homology of human and equine influenza A viruses and suggest the possibility of their recombination in nature.*

Although the source of interpandemic outbreaks of influenza in man is probably man himself (*1*), the origin of the pandemics remains a mystery. Studies of the pandemic of 1957 reinforced earlier indirect evidence that pandemics are associated with the emergence of antigenically novel influenza A viruses. The sudden appearance of such viruses, without evidence of continuous mutation from prior human strains, has suggested that they may have come from animal reservoirs (*2*). Serologic evidence points to the etiologic role of a virus similar to the swine influenza virus of the 1918 pandemic (*3*), although the virus may have been transmitted from man to swine rather than in the other direction (*4*). Recently, new influenza A viruses have been isolated from several avian species (*5*), and one such strain is antigenically related to human influenza A_2 subtype virus (*6*). Two antigenically distinct equine influenza A viruses, equine 1 and equine 2 were isolated in 1956 and 1963, respectively. One (A/equine 2) may be related antigenically to a human influenza A virus of the last century (*7*) and is capable of infecting man (*8*).

Genetic recombination of human and animal influenza A viruses in nature could provide an immediate exchange of markers and, in effect, an instant "multistep mutation." Hybridization of human (*9, 10*) and of human and avian strains incorporating antigens from each parent (*11*) has been accomplished in the laboratory. I now describe a hybrid derived from influenza A/equine 1/56 virus and an A_2 recombinant virus (X-1L), the latter having been derived from the human strains A_0/NWS/33 and A_2RI/5$^+$/57 (Fig. 1). Recombinant X-1L was selected for recombination with the equine virus because its capacity to form plaques in a unique cell line, clone 1-5C-4 (*12*), and its high neuraminidase activity (E) provided distinctive markers potentially useful in the isolation of recombinants of equine serotype.

Chick embryos 10 days of age (four per group) were infected via the allantois with a mixture of 10^4, EID_{50} (egg infective doses 50 percent effective) of A/equine 1 and X-1L viruses. Control groups received each virus singly in equivalent dosage. After a 40-hour incubation period at 35°C, hemagglutinating virus demonstrated in allantoic fluid harvest was inoculated [diluted in decimal series (10^{-1} to 10^{-6})] onto clone 1-5C-4 cell monolayers in plastic petri dishes (*12*). Monolayers were then covered with a layer of agar containing A_2e antibody selective against the major (A_2) antigen of X-1L, but not against its neuraminidase (E) (Fig. 1). This antiserum was produced by immunization of rabbits with a recombinant, X-9, that has A_2 hemagglutinin but A_0 neuraminidase (*10*). One 0.5-mm plaque was detected in a dish inoculated with the 10^{-2} dilution. A plug of agar immediately overlying the plaque was inoculated into eggs and clone 1-5C-4 monolayers, for passage and isolation of an equine E recombinant (Fig. 1). Poorly visible plaques were only sporadically produced in subsequent passage so that further cloning and isolation of this recombinant (X-15) were carried out in eggs by the limiting dilution technique. The methods used for viral neuraminidase assay and for characterization of the hemagglutinin by hemagglutination inhibition have been described (*13, 14*). Individual allantoic fluids positive for hemagglutinating virus were tested for viral neuraminidase activity with a fetuin substrate. Fluids with high activity were retested in the presence of antiserum specific for A_2 neuraminidase (*15*). Viruses in those fluids in which activity was inhibited were identified in hemagglutination inhibition tests as being simi-

Table 1. The antigenic relation of recombinant X-15 to its parental viruses, and to the human influenza A_0 and A_2 prototype viruses NWS and RI/5 and their recombinants. Results are expressed as the reciprocals of serum dilutions at the endpoint of hemagglutination inhibition titrations. The symbols for the viral antigens are as follows: the first symbol (Eq, A_0, A_2) represents the hemagglutinin protein (that is, the major antigen by which subtype definition is made); the second symbol (eq, e, E) is the viral neuraminidase. The antiserum designations are by specific strain. In addition, anti-E represents antiserum specific for influenza A_2 viral neuraminidase (E).

Virus	Antigens	Titers of antiserums									
		Equine 1	X-1L	X-15	NWS	X-7	X-12	X-9	RI/5$^+$	RI/5$^-$	anti-E
Equine 1	Eq eq	*1024*	<4	5120	4	<4	<4	<4	4	<4	<10
X-1L*	A_2 E	16	*2048*	2560	16	32	160	320	1024	512	500
X-15	Eq E	4096	512	*5120*	8	64	2048	8	128	512	1024
NWS	A_0 e	8	16	<4	*512*	2048	2048	16	<4	<4	<10
X-7*	A_0 E	<4	16	<4	1024	*4096*	4096	<4	8	8	20
X-12*	A_0 E	<4	8	<4	512	4096	*4096*	<4	8	4	64
X-9*	A_2 e	<4	64	<4	4	<4	<4	*1280*	512	64	<10
RI/5$^+$	A_2 E	16	1024	128	16	32		320	*512*	1024	100
RI/5$^-$	A_2 E	<4	256	<4	<4	4	40	160	512	*320*	<10

* Recombinant of A_0/NWS and A_2RI/5.

lar to equine-virus and, hence, were considered to be recombinant for equine hemagglutinin protein and for A_2 neuraminidase (equine E).

After the recombinant (X-15) was cloned, a large batch of virus was prepared for chemical studies. The virus, after purification by centrifugation in a sucrose density gradient, was disrupted with sodium dodecyl sulfate, and its proteins were separated by electrophoresis on cellulose acetate (*13*). The neuraminidase extracted from fraction 3 of the cellulose acetate strip (*13*) was identified as that of influenza A_2, and thus had been derived from the X-1L parent. No protein was demonstrable in this region in the case of equine 1 virus, and the electrophoretogram differed markedly from that of X-15.

The results of antigenic analysis by

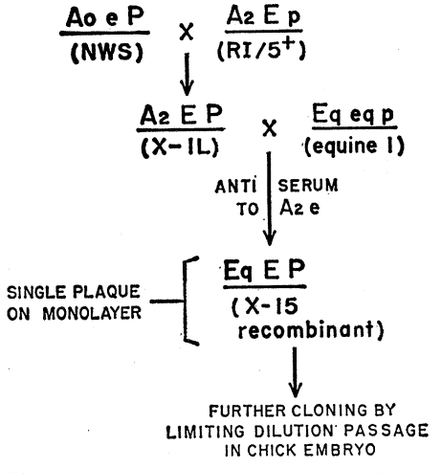

Fig. 1. Derivation of recombinant X-15. A_0, A_2, and Eq represent the major (hemagglutinin) antigens of the viruses; e, E, and eq indicate the neuraminidase components; P indicates the capacity and p the incapacity of virus to form plaques in clone 1-5C-4 cell monolayer cultures. The virus strain designations are parenthesized. The antiserum to A_2e selectively neutralizes parental virus or recombinant progeny containing the A_2 hemagglutinin.

hemagglutination inhibition of the equine E recombinant, X-15, and its parental and related viruses are presented in Table 1. Antiserums were produced in rabbits by the intravenous injection of 10^4 to $10^{5.6}$ hemagglutinating units of partially purified virus, and a single booster injection was given at 15 to 40 days. Serums were inactivated by heating to 56°C followed by treatment with sodium periodate. The X-15 virus and its antiserum react to high titer with both parental viruses or their antiserums; little cross reaction of equine 1 and X-1L was detected. Also, X-15 virus was inhibited to some degree by antiserums to six viruses deriving antigens from the A_0 and A_2 human subtypes, in contrast to equine 1 virus which was inhibited only by low dilutions of antiserums to A_0/NWS and A_2/RI/5$^+$. Antiserums to equine 1 and X-15 viruses did not differ significantly in their inhibition of the human viruses except in the case of the X-1L parent of X-15.

The X-15 virus was significantly inhibited only with antiserums to human viruses containing the A_2 neuraminidase (E) regardless of their hemagglutinin moiety. However, in hemagglutination tests with the E-containing viruses X-7 and X-12 such inhibition was observed only with low dilutions of antiserum [note reactions with antiserums to RI/5$^+$, RI/5$^-$ and E (anti-E) in Table 1]. The reaction is not dependent on the possession by X-15 of an A_2 antigen other than E, as is shown by the low activity of X-9 (A_2e) antiserum with X-15 virus. Finally, antiserum to isolated A_2 neuraminidase inhibits X-15 hemagglutination at high dilution, so that inhibition of hemagglutination with X-15 (and the parental X-1L) appears to involve antibody attachment at the enzyme site on the virion. In neutralization tests in the chick embryo, X-15 reacts only with equine 1 or homologous antiserum so that its major surface antigen clearly is derived from equine 1 virus.

Other equine E recombinants derived from crosses of equine 1 and X-7 and X-12 viruses have properties similar to those of X-15, despite the relative insusceptibility of the parental viruses to hemagglutination inhibition with antiserum to neuraminidase (unlike X-1L). Thus, it appears that A_2 neuraminidase, when structurally associated with equine 1 hemagglutinin in the envelope of the virion, may be so situated that antibody to the enzyme can effectively prevent attachment of the virus to red blood cells, yet not effect neutralization.

In conclusion, genetic homology of A/equine 1 and influenza A viruses of human origin has been demonstrated by recombination, with the production of stable, antigenically distinct hybrids deriving proteins from both parents. Furthermore, antigenic analysis of the parental and other viruses has demonstrated slight but definite cross reactivity of A/equine 1 with human influenza A viruses.

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References and Notes

1. J. Hayslett, J. R. McCarroll, E. Brady, K. Deuschle, W. McDermott, E. D. Kilbourne, *Amer. Rev. Resp. Dis.* **85**, 1 (1962).
2. C. H. Andrewes, in *Perspectives in Virology*, M. Pollard, Ed. (Wiley, New York, 1959), p. 184.
3. F. M. Davenport, A. V. Hennessy, T. Francis, Jr., *J. Exp. Med.* **98**, 641 (1953).
4. P. P. Laidlaw, *Lancet* **1935-I**, 1118 (1935); R. E. Shope, in *The Harvey Lectures, ser. 31*, New York Academy of Medicine (Williams and Wilkins, Baltimore, 1935–1936), p. 183.
5. H. G. Pereira, B. Tumova, V. G. Law, *Bull. World Health Organ.* **32**, 855 (1965).
6. H. G. Pereira, B. Tumova, R. G. Webster, *Nature* **215**, 982 (1967).
7. E. Minuse, J. L. McQueen, F. M. Davenport, T. Francis, Jr., *J. Immunol.* **94**, 563 (1965).
8. J. A. Kasel, R. H. Alford, V. Knight, G. H. Waddell, M. M. Sigel, *Nature* **206**, 41 (1965).
9. E. D. Kilbourne and J. L. Schulman, *Trans. Ass. Amer. Physicians* **78**, 323 (1965).

10. E. D. Kilbourne, F. S. Lief, J. L. Schulman, R. I. Jahiel, W. G. Laver, in *Perspectives in Virology*, M. Pollard, Ed. (Academic Press, New York, 1967), p. 87.
11. B. Tumova and H. G. Pereira, *Virology* **27**, 253 (1965).
12. A. Sugiura and E. D. Kilbourne, *ibid.* **26**, 478 (1965).
13. W. G. Laver and E. D. Kilbourne, *ibid.* **30**, 493 (1966).
14. S. C. Wong and E. D. Kilbourne, *J. Exp. Med.* **113**, 95 (1961).
15. E. D. Kilbourne, W. G. Laver, J. L. Schulman, R. G. Webster, *J. Virol.*, in press.
16. Conducted under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board and supported by U.S. Army Medical Research and Development Command, under research contract DA-49-193-MD-2795; in part by PHS research grant AI-01595 from the NIAID; and also by the Health Research Council of the City of New York under contract No. U-1023.
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Aspirin: Dissolution Rates of Two Polymorphic Forms

Abstract. *Two polymorphic forms of aspirin were characterized; the rates of dissolution of single crystals and of tablets were studied. One form dissolves 50 percent faster than the other.*

In evaluating the drug action (*1*) of different formulations of aspirin, crystal modification and the possible effect of polymorphism on drug availability (*2*) have frequently been disregarded. I now report that the rates of dissolution of two polymorphic forms of aspirin were different, regardless of whether the measurement was made from a single crystal or from a tablet.

Polymorph I was prepared by slow crystallization at room temperature from a saturated solution of commercial aspirin (U.S.P.) in 95 percent ethanol. The melting point (determined with a Kofler hot-stage microscope) was 143° to 144°C, a value which is in agreement with the monoclinic crystal structure determined by Wheately (*3*). Polymorph II was prepared by slow crystallization from a saturated solution of commercial aspirin (U.S.P.) in *n*-hexane at room temperature and melted at 123° to 125°C. Both forms meet the U.S.P. specifications.

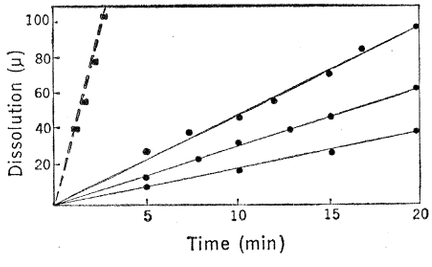

Fig. 1. Single-crystal dissolution as a function of time. Circles, form I; rectangles, form II.

X-ray diffraction (powder method) with nickel-filtered copper radiation and infrared spectra (Nujol mull) were used to characterize the two crystalline forms. Differences in x-ray diffraction patterns and in absorption spectra (not shown here) indicated different arrangements of aspirin molecules in the crystal lattice of each form.

The linear rate of dissolution was measured by a direct optical method (*4*). A single crystal of aspirin 400 to 600 μ was fixed into a rubber slit and placed in a jacketed dissolution cell. The cell was filled with 400 ml of distilled water and kept at a constant temperature of 30°C. Stirring was maintained at 150 rev/min by a synchronized motor. The distance between the two parallel faces of the mounted crystal and the boundary movement across each face were measured as a function of time. Measurements were done by means of a microscope fitted with a filar micrometer. The optical system could differentiate boundary movement in the order of 2 μ, and dissolution data were reproducible within $\pm$ 5 percent for ten determinations. Figure 1 demonstrates the degree of dissolution in microns as a function of time for the two crystal forms. The other two axes in form II could not be measured owing to limitation of the optical system to measure with accuracy the boundary movement in a thin needle-like crystal.

With slight modifications, the dissolution rate of aspirin tablets were studied with the same apparatus. Aspirin tablets from each polymorphic form were prepared under the same pressure and having the same diameter, 1.88 cm; no fillers, lubricants, or antiadhesives were used. The tablet was then placed at one end of a short glass tube having the same inside diameter as the diameter of the tablet, the other end of the tube was filled with molten, white beeswax and left to set; wax was used to hold the tablet.

At zero time, the tube was introduced into the dissolution cell containing 400 ml of distilled water at 30°C. Samples of the water were then pipetted out at specified time intervals and assayed spectrophotometrically at 282 nm. The assay was reproducible within $\pm$ 2 percent for six determinations. Figure 2 shows the amount (μg/ml) dissolved as a function of time for forms I and II. Their initial slopes are 0.862 and 1.350, respectively.

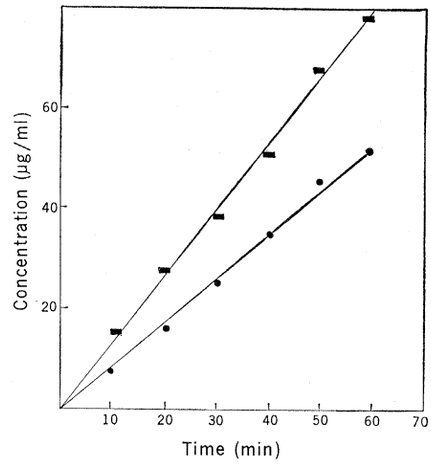

Fig. 2. Dissolution rates of aspirin from tablet. Circles, form I; rectangles, form II.

In the single-crystal comparison, dissolution occurred with different velocities along the different axes (anisotropic). The average rate for form II was evidently greater than that for form I, in spite of the unknown contribution of the other two axes in the dissolution process. Tablets prepared from form II dissolved about 50 percent faster than those of form I, and the results obtained are in agreement with studies on single crystals. From this limited study, it appears that polymorph II has a greater thermodynamic activity and exhibits a higher dissolution rate than polymorph I. Polymorph I was very similar to commercial aspirin (U.S.P.) used in this study, in dissolution rate from tablet and in x-ray diffraction pattern, the only difference being that it was grown from ethanol in nearly perfect crystals, suitable for single-crystal dissolution, while commercial aspirin U.S.P. consisted of crushed, irregularly shaped crystals.

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References and Notes

1. S. V. Lieberman, S. R. Kraus, J. Murray, J. H. Wood, *J. Pharm. Sci.* **53**, 1486 (1964); S. N. Lieberman and J. H. Wood, *ibid.*, p. 1492; C. Pfeiffer, L. Goldstein, H. Murphree, M. Hopkins, *ibid.* **56**, 1338 (1967).
2. R. Tawashi, "Some factors associated with the dissolution of crystalline drugs," *Int. Congr. Pharm. Sci., Montpelier*, September 1967.
3. P. J. Wheately, *J. Chem. Soc.* **1964**, 6036 (1964).
4. W. Higuchi, P. Bernardo, C. Metha, *J. Pharm. Sci.* **56**, 200 (1967); P. Bernardo, thesis, University of Michigan (1966); W. Higuchi and R. Tawashi, in preparation.
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