QUANTITATIVE ASPECTS OF HOMOLOGOUS AND HETEROLOGOUS ACTIVE IMMUNITY TO STRAINS OF THE VIRUS OF EPIDEMIC INFLUENZA*

By MONROE D. EATON, M.D., AND HAROLD E. PEARSON, M.D.

(From the Research Laboratory of the California State Department of Public Health, Berkeley)

(Received for publication, August 9, 1940)

In immunization against epidemic influenza, one of the most important problems is the production of effective resistance to heterologous strains of influenza virus. Recent work has emphasized qualitative differences between strains of the virus (1–5). Other observations have indicated the existence of relationships determined by various factors which affect the specificity of the immunity, such as the route (3, 6–8) and the number of inoculations (2, 5, 9), the interval after inoculation at which the immunity test is done (1, 5), the degree of involvement of the susceptible respiratory epithelium (6), the antigenic specificity of the strain (1, 3), and the amount of virus used for immunization.

In the present work an attempt has been made to evaluate quantitatively the relationships of several strains of influenza virus as measured by active immunity in mice. In previous quantitative work on heterologous active immunity, animals have usually been immunized with a constant amount of virus and tested for immunity with varying amounts inoculated intranasally (2, 6). In the present work the vaccinating dose has been varied and the test dose kept constant in order to measure the relative amounts of virus given by the intraperitoneal or by the intranasal route which are required to produce a comparable immunity to homologous and heterologous strains. An attempt has been made to distinguish between factors which tend to increase heterologous immunity more than homologous immunity, and those which merely bring about a quantitative increase of similar degree in the immunity to all strains. The results indicate that the most important factor in cross-immunity is the size of the vaccinating dose.

* These studies were made under the auspices, and with the support, of the International Health Division of The Rockefeller Foundation cooperating with the California State Department of Public Health.

Downloaded from http://rupress.org/jem/article-pdf/72/6/635/1182058/635.pdf by guest on 23 September 2021

This is probably dependent on the amount of antigenic component which different strains have in common.

A detailed study has been made of five strains of influenza virus. The strains PR8, Melbourne, and Philadelphia belong to the so called "intermediate" class and have definite antigenic relationships (1, 3) while the strains W.S. and swine have been classified as "specific" (1, 2).

Materials and Methods

The material used for immunization was as follows: (*a*) Mouse passage strains PR8, W.S., Melbourne, and swine as suspensions of infected mouse lung in broth. (*b*) Ferret passage strains PR8 and Philadelphia as suspensions of infected ferret lung in broth. (*c*) The chick embryo tissue culture strain of swine influenza virus which had been carried by Dr. Francis from infected swine lungs directly to tissue culture.

The amount of virus used for immunization was measured in terms of intranasal M.L.D. as previously described (10), and mice in groups of 8 received 0.5 cc. each intraperitoneally, or 0.05 cc. intranasally, of serial tenfold dilutions up to and including dilutions which contained an amount of virus too small to produce immunity to the homologous strain.

The ferret passage and tissue culture strains were somewhat attenuated for mice (11), and they had much more immunizing effect per intranasal M.L.D. than did the highly virulent mouse passage strains. This made it possible to study the effects on homologous and heterologous immunity of intranasal inoculation with various sublethal quantities of virus.

Two weeks after the last immunizing inoculation, the mice were tested for active immunity by intranasal instillation in a volume of 0.05 cc. of between 1 and 10 M.L.D. of the homologous and heterologous strains. In all of these tests for immunity previously titrated suspensions of highly virulent mouse passage strains of PR8, W.S., Melbourne, Philadelphia, and swine were used. Deaths or survivals with or without lung lesions in each group of mice following the test were recorded as percentage scores (10) as given by the following expression:

$$\text{Percentage score} = \frac{5a + 4b + 3c + 2d + 1e}{5T}$$

Where a represents the number of mice dead, b, c, d, and e represent the number of mice surviving with ++++, +++, ++, and + lung lesions respectively, and T is the total number of mice in the group, including those surviving without lung lesions.

Measurement of the Intraperitoneal Minimal Immunizing Dose for Homologous and Heterologous Strains

Mice were immunized by one intraperitoneal injection of 0.5 cc. of the active mouse passage strains W.S., PR8, or Melbourne. In a second experiment 3 inoculations, 10 days apart, were given in order to compare the effect of 1 and 3 injections. Tests for immunity were done as described in the previous section.

Downloaded from http://rupress.org/jem/article-pdf/72/6/635/1182058/635.pdf by guest on 23 September 2021

The results are presented in Table I. The percentage scores of control groups of mice which received less than the minimal amount of virus necessary to immunize against the homologous strain will be seen in the last two columns of the table. When the scores of groups of mice receiving larger amounts of virus for immunization were less than half the scores in the corresponding control group, some active immunity was considered to be indicated. When there was no indication of immunity, the percentage score in the table has been enclosed in parentheses.

TABLE I

Immunity after Intraperitoneal Injection of Active Virus: Effect of Amount of Virus and Number of Injections

Vaccinated with strain	Tested with strain	Percentage scores* of groups of mice immunized with					
		1 × 10,000 M.L.D.	3 × 1,000 M.L.D.	1 × 1,000 M.L.D.	3 × 100 M.L.D.	1 × 100 M.L.D.	3 × 10 M.L.D.
W.S.	W.S.	3	0	27	12	(75)	(80)
"	PR8	3	12	(65)	(52)	(65)	(98)
"	Melb.	31	22	(50)	45	(100)	(95)
"	Swine	30	13	(60)	(40)	(73)	(53)
PR8	W.S.	26	25	(53)	(75)	(88)	(78)
"	PR8	3	0	12	8	(56)	(72)
"	Melb.	12	0	(43)	(25)	(46)	(40)
"	Swine	40	31	(63)	(42)	(77)	(47)
Melb.	W.S.	32	30	(90)	(58)	(100)	(100)
"	PR8	20	10	(78)	(100)	(100)	(89)
"	Melb.	0	2	12	17	(76)	(55)
"	Swine	(78)†	(60)	(72)	(83)	(78)	(88)

* In this and succeeding tables percentage scores enclosed in parentheses indicate absence of immunity. Expression 1 × or 3 × before number of M.L.D. indicates number of immunizing injections.

† Immunity to the swine strain was obtained with one intraperitoneal dose of 100,000 M.L.D. of Melbourne.

In general, the amount of active virus required for immunity to the heterologous strains was approximately 10 times the minimal immunizing dose for the homologous strain. In mice immunized with the strain Melbourne and tested with swine influenza virus an exception was noted. Here the ratio of homologous to heterologous minimal immunizing doses was 1 to 100. When mice were immunized by intraperitoneal inoculation of the swine influenza virus and tested with human strains, this ratio was still larger, as will be shown in a later section.

With 3 intraperitoneal injections, about one-third as much total virus was required to produce an immunity equivalent to that obtained with 1

Downloaded from http://rupress.org/jem/article-pdf/72/6/635/1182058/635.pdf by guest on 23 September 2021

inoculation, but the ratio of homologous to heterologous minimal protective doses remained the same. The results indicated that immunity to both homologous and heterologous strains was increased to the same degree by repeated immunizing injections.

With single doses it was necessary to inject 10,000 intranasal M.L.D., in order to produce some heterologous immunity. This amount of virus will produce lung lesions in some of the mice after injection into the peritoneal cavity, and 100,000 M.L.D. given by the same route, although producing solid heterologous immunity in the survivors, usually killed some of the mice with complete consolidation of the lungs. This finding suggested that the production of effective heterologous active immunity in mice might be dependent on infection of the lung tissue.

TABLE II

Relative Amounts of Active and Inactive Influenza Virus Required for Heterologous Immunity after One Intraperitoneal Injection

Tested with strain	Mice immunized with PR8 mouse lung						
	Active			Inactive			
	10,000 units*	1,000 units	100 units	1,000,000 units	100,000 units	10,000 units	1,000 units
PR8	3	12	(56)	0	0	20	(90)
W.S.	26	(53)	(88)	25	35	(80)	—
Melb.	12	(43)	(46)	5	10	(90)	—

* In this table the term "unit" is used to denote the active or inactive equivalent of one intranasal M.L.D.

To investigate this point, experiments were done with formalinized inactive preparations. The results presented in Table II indicate that heterologous immunity may be induced by intraperitoneal injection of inactive virus. This agrees with the results of Oakley and Warrack (6). The ratio of homologous to heterologous minimal immunizing doses is again 1 to 10, or the same as with active virus. However, 10 to 100 times as much formalinized inactive virus is required to produce a degree of immunity comparable to that obtained with active virus (10).

Homologous and Heterologous Immunity after Intranasal Inoculation of Mice with Ferret Passage Strains

It was shown in a previous study (11) that certain ferret passage strains of human influenza virus were capable of producing solid homologous immunity in mice inoculated intranasally with a very small fraction of an M.L.D. As may be seen in Table III, solid immunity to heterologous human strains and swine influenza virus was also obtained by intranasal inoculation

Downloaded from http://rupress.org/jem/article-pdf/72/6/635/1182058/635.pdf by guest on 23 September 2021

of mice with 1/100 M.L.D. of the ferret passage strain Philadelphia. Significant immunity to four human strains and partial immunity to the swine influenza virus followed intranasal instillation of 1/10,000 M.L.D.

The same preparation after intraperitoneal inoculation gave some immunity to the heterologous strains PR8 and Melbourne, but none to W.S. or swine. It appears, therefore, that the strain Philadelphia has a rather non-specific antigenic composition and that extension of the immunity to other strains is most pronounced after intranasal inoculation.

In contrast to these results with the strain Philadelphia, intranasal inoculation of mice with sublethal doses of the ferret passage strain PR8 gave an immunity which was not much less specific than that obtained by

TABLE III

*Heterologous Immunity after Intranasal and Intraperitoneal Inoculation with Philadelphia Ferret Passage Virus**

Tested with	Percentage scores of groups of mice inoculated						
	Once I.N. with				Once I.P. with		
	1/100 M.L.D.	1/1,000 M.L.D.	1/10,000 M.L.D	1/100,000 M.L.D.	10 M.L.D.	1 M.L.D.	1/10 M.L.D.
Phila.	1	6	16	(93)	—	40	(77)
PR8	7	3	11	(75)	—	20	(77)
Melb.	3	—	16	(95)	35	42	(90)
W.S.	3	3	19	(75)	(75)	(75)	(95)
Swine	5	42	47	(80)	(100)	(100)	(100)

* With this strain one intranasal M.L.D. for mice was 0.05 cc. of a 10 per cent suspension of ferret lung.

I.N. = intranasally. I.P. = intraperitoneally.

the intraperitoneal route of injection. The results presented in Table IV show that mice receiving 1/10,000 of an M.L.D. developed a solid immunity to the homologous strain and to the strain Melbourne, but none to W.S. and swine. With the exception of the cross-immunity to Melbourne, a similar result was obtained by intraperitoneal inoculation of 1,000 intranasal M.L.D. of the mouse passage strain (Tables I and II). The immunity was not increased or broadened appreciably by repeated intranasal inoculation with small amounts of virus, but a single larger intranasal dose of 1/100 M.L.D. gave solid immunity to the three heterologous strains.

Cross-Immunization with Swine Influenza Virus: Effect of Route and Number of Injections

It has been noted by other investigators (7, 8) that mice and ferrets recovered from infection with the swine influenza virus are actively immune

Downloaded from http://rupress.org/jem/article-pdf/72/6/635/1182058/635.pdf by guest on 23 September 2021

to human strains. On the other hand, recovery from infection with one human strain does not always confer immunity to another human strain either in ferrets (4) or in mice (3). Because of this discrepancy in the published results, it was considered desirable to reinvestigate, by the application of quantitative methods, the immunity to human strains produced by intraperitoneal and intranasal inoculation with swine influenza virus.

TABLE IV

Heterologous Immunity after Intranasal Inoculation with PR8 Ferret Passage Virus

Tested with	Percentage scores of groups of mice inoculated with						
	1 × 1/100 M.L.D.	1 × 1/1,000 M.L.D.	3 × 1/1,000 M.L.D.	1 × 1/10,000 M.L.D.	3 × 1/10,000 M.L.D.	1 × 1/100,000 M.L.D.	3 × 1/100,000 M.L.D.
PR8	5	0	0	5	0	(74)	(100)
Melb.	0	0	—	10	—	(75)	—
W.S.	5	(55)	25	(73)	(50)	(100)	(54)
Swine	8	(45)	(43)	(77)	(60)	(83)	(62)

TABLE V

Ratio of Homologous to Heterologous Minimal Immunizing Dose in Mice Immunized with Swine Influenza Virus

Tested with strain	Homologous minimal immunizing doses required for immunity in mice receiving				
	Swine M.L.* once I.P.	Swine M.L. thrice I.P.	Swine T.C.* thrice I.P.	Swine T.C. once I.N.	Swine T.C. thrice I.N.
Swine	1	1	1	1	1
W.S.	1,000	1,000	1,000	10	1†
PR8	1,000	1,000	1,000	10	1†

Dilution and number of M.L.D. in homologous minimal immunizing dose:
Swine mouse lung I.P. 0.5 cc. of 10^{-4} or 1,000 M.L.D.
Swine tissue culture I.P. 0.5 cc. of 10^{-3} or 1 M.L.D.
Swine tissue culture I.N. 0.05 cc. of 10^{-5} or 1/1,000 M.L.D.
* M.L. = mouse lung. T.C. = tissue culture.
† More lesions at each immunizing dilution than with homologous strain.

Mice received 1 and 3 inoculations of various amounts of swine tissue culture and mouse passage strains. The least amount of virus required for immunity against the homologous swine strain in each series of tests was taken as the homologous minimal immunizing dose for the route and number of inoculations given.

The results in Table V are presented in terms of the number of homologous (swine) minimal immunizing doses required for effective heterologous immunity to the two human strains W.S. and PR8. When the intra-

Downloaded from http://rupress.org/jem/article-pdf/72/6/635/1182058/635.pdf by guest on 23 September 2021

peritoneal route of inoculation was used, the ratio in each case was approximately 1 to 1,000. Intranasal inoculation produced a much less specific immunity as indicated by the fact that the minimal amount of swine virus required for protection against the human strains was only 10 times that required for immunity to the swine strain. Three intranasal inoculations with a dilution of 10^{-5} of the swine tissue culture virus protected all mice against death when tested with swine and human strains, but lung lesions were more frequent and extensive in the mice tested with the strains PR8 and W.S.

DISCUSSION

The results of the experiments on active immunization of mice by the intraperitoneal route indicate that the relationship between certain human strains of epidemic influenza virus is determined by common antigens which are present in the proportion of 1 part in the heterologous strain to about 10 parts in the homologous strain. This follows from the observation that effective heterologous immunity is obtained by injecting 10 times the homologous minimal immunizing dose.

On the same basis it appears that the amount of human strain antigen in the swine influenza virus is approximately one-thousandth of the quantity of this antigen in the strains PR8, W.S., or Melbourne. Conversely, these human strains contain one-tenth to one-hundredth as much swine antigen as the swine strain. The quantitative antigenic relationships between swine and human strains are therefore not exactly reciprocal, a fact originally pointed out by Francis and Shope (12) as a result of their experiments on the production of neutralizing antibodies.

The quantitative interpretation just outlined is also in accord with other observations. Repeated intraperitoneal inoculation increases homologous and heterologous immunity to the same degree. The ratio between homologous and heterologous minimal immunizing doses is not altered when inactive virus is used, but enormous amounts of material are required for immunity. The development of lung lesions does not appear to be essential to heterologous immunity. Taken as a whole, the results are analogous to immunization with serologically and chemically related substances containing various proportions of antigenic groups. The degree of immunity to any one antigenic group is proportional to the amount of that group injected and is related to the repetition of the stimulus.

Since the degree of infection-immunity in mice also seemed to be related to the intranasal immunizing dose, and since multiplication of the virus would produce a uniform increase in all groups of the antigenic pattern, it

Downloaded from http://rupress.org/jem/article-pdf/72/6/635/1182058/635.pdf by guest on 23 September 2021

might be expected that the ratio between homologous and heterologous minimal immunizing doses would be the same as for the intraperitoneal route.

The experiments on intranasal immunization with the ferret passage strains PR8 and Philadelphia showed that with minimal doses there was relatively more cross-immunity to closely related strains (PR8, Melbourne, and Philadelphia) than after immunization by the intraperitoneal route. However, the strain PR8 exhibited, with respect to W.S. and swine, a specificity which was independent of the route of inoculation. Strain specificity was also apparent after intranasal immunization of mice with 1/1,000 M.L.D. of swine influenza virus, but a tenfold increase in the dose produced effective cross-immunity to human strains. By the intraperitoneal route 1,000 homologous minimal immunizing doses were required for heterologous immunity. In this connection it is of some interest to note that the antigenic differences between the two human strains PR8 and W.S., as judged by quantitative intranasal immunity tests, were somewhat more pronounced than the differences between swine and human strains (Tables IV and V).

The broader immunity obtained by intranasal inoculation, as compared with intraperitoneal immunization, appeared to be due to factors other than the antigenic composition of the virus. The possibility of a non-specific local resistance following infection must be considered, but if this is the correct explanation, it is obvious from the results just discussed that some strains increase this local resistance while others have a negligible effect.

The experiments on immunization of mice raise certain questions as to the quantity of virus necessary for protection of human beings. On the basis of body weight, the amount of tissue containing active or inactive virus which is required to produce effective heterologous immunity in mice by intraperitoneal inoculation is at least 1,000 times any dosage yet attempted in man. On the other hand, heterologous immunity in mice was obtained by intranasal inoculation with relatively minute doses of partially attenuated virus. Similar observations by Burnet (3) have indicated the possibilities of intranasal inoculation, and Francis (13) has recently published observations on intranasal inoculation of human beings with the tissue culture strain PR8.

SUMMARY

When mice are immunized by one intraperitoneal inoculation with active or inactive influenza virus (strain PR8, W.S., and Melbourne) the quantity

Downloaded from http://rupress.org/jem/article-pdf/72/6/635/1182058/635.pdf by guest on 23 September 2021

required for protection against heterologous strains is about 10 times the homologous minimal immunizing dose. Three injections increase the immunity to all strains, but the ratio between the homologous and heterologous minimal immunizing dose is not altered.

Swine influenza virus given intraperitoneally fails to immunize against human strains unless the quantity injected is 1,000 times the minimal amount required for homologous immunity.

Intranasal immunization of mice with 1/100 M.L.D. of attenuated ferret passage strains PR8 and Philadelphia, or the tissue culture strain of swine influenza, gives a solid resistance to infection with heterologous strains. When smaller amounts of virus are given intranasally, strain specificity becomes more apparent, and with minimal doses the immunity may be effective only against the homologous and closely related strains.

BIBLIOGRAPHY

1. Smith, W., and Andrewes, C. H., *Brit. J. Exp. Path.*, 1938, **19,** 293.
2. Francis, T., Jr., and Magill, T. P., *Brit. J. Exp. Path.*, 1938, **19,** 284.
3. Burnet, F. M., *Australian J. Exp. Biol. and Med. Sc.*, 1937, **15,** 369.
4. Horsfall, F. L., Jr., and Lennette, E. H., *J. Bact.*, 1940, **39,** 56.
5. Taylor, R. M., Dreguss, M., and De Ritis, F., *Am. J. Hyg.*, Section B, 1940, **31,** 36.
6. Oakley, C. L., and Warrack, G. H., *J. Path. and Bact.*, 1940, **50,** 37.
7. Smith, W., Andrewes, C. H., and Laidlaw, P. P., *Brit. J. Exp. Path.*, 1935, **16,** 291.
8. Shope, R. E., *J. Exp. Med.*, 1935, **62,** 561.
9. Stuart-Harris, C. H., Andrewes, C. H., and Smith, W., *Great Britain Med. Research Council, Special Rep. Series, No. 228*, 1938, 133.
10. Eaton, M. D., *J. Immunol.*, 1940, **39,** 43.
11. Eaton, M. D., and Beck, M. D., *J. Immunol.*, 1940, **39,** 57.
12. Francis, T., Jr., and Shope, R. E., *J. Exp. Med.*, 1936, **63,** 645.
13. Francis, T., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43,** 337.

Downloaded from http://rupress.org/jem/article-pdf/72/6/635/1182058/635.pdf by guest on 23 September 2021