STUDIES ON PNEUMONIA VIRUS OF MICE (PVM)

II. Immunological Evidence of Latent Infection with the Virus in Numerous Mammalian Species*

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The unpredictable occurrence of latent viruses in apparently normal laboratory animals may be responsible for confusion and errors in the interpretation of the results of experimental procedures. The frequency with which latent viruses are encountered appears to be greatly increased when serial passages are carried out, as would be anticipated from theoretical considerations.

During the past few years, numerous papers have appeared in which viruses encountered in the respiratory tract of seemingly healthy mice were described. Some of these latent agents cause pneumonia. One of them, which we have called pneumonia virus of mice (PVM) (1), is strikingly different in a number of its characteristics from other viruses also capable of inducing pneumonia, which have been studied in detail. These characteristics were enumerated in the preceding paper (2). This virus was recognized first in 1938 in New York during the course of studies with Swiss mice. It has been encountered since in similar mice in California (3) and Minnesota (4). Further investigations carried out in this laboratory revealed that pneumonia virus of mice could be obtained from numerous different stocks of mice, and that latent infection by it was not confined solely to Swiss mice.

During 1942 evidence began to accumulate which indicated that this virus, or one antigenically similar to it, was present in a latent state in a number of mammalian species other than the mouse. Pearson and Eaton (3) had reported the occasional occurrence of a similar virus in hamsters. When it was noted in this laboratory that various apparently normal animals of several species possessed neutralizing antibodies against pneumonia virus of mice, a systematic study of the problem was undertaken. Recently Eaton and van Herick (5) have reported that rabbits and cotton rats showed evidence of infection by a latent virus related to pneumonia virus of mice.

It is the purpose of this paper to show that of nine mammalian species studied, each gave evidence of infection by an agent antigenically similar to, if not

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identical with, pneumonia virus of mice. It will also be shown that the frequency of infection by this agent in normal animals was correlated with certain seasons. Evidence will be presented which indicates that certain non-specific stimuli are capable of unbalancing an equilibrium between this latent agent and its natural hosts, thereby inducing an inapparent infection and the development of specific immunity.

Methods

Animal Species.—Nine mammalian and one avian species were studied during the period from June, 1942, to March, 1945. The pertinent data regarding these species are as follows: Fully grown chickens (Plymouth Rock) were obtained from commercial sources in New York City; chimpanzee sera were made available by Dr. Howard Howe, Baltimore, Maryland; cotton rats (*Sigmodon hispidus hispidus* and *Sigmodon hispidus littoralis*) were obtained from the Michigan State Department of Health, Lansing, Michigan; guinea pigs (albino) were obtained from the stock bred at The Rockefeller Institute; hamsters (*Cricetus auratus*) were obtained from one commercial breeder in New York; wild mongoose (*Mungos birmanicus*) were trapped in Puerto Rico and their sera were made available by Dr. A. E. Feller, Fort Bragg, North Carolina; monkey sera (*Macaca mulatta*) were made available by Dr. Max Theiler, New York City; mice (albino Swiss) were obtained from two commercial breeders in the vicinity of New York City; and rabbits (mixed hybrid) were obtained from the stock bred at The Rockefeller Institute. Human sera from normal persons at a large naval training station in New York City were made available by Captain John B. Farrior (MC) U. S. N.

During the period of study, all chickens and rabbits were kept in a separate animal room, to which no other laboratory animals were admitted. Monkeys were kept in another separate animal room into which other laboratory animals were only rarely admitted. Both of these animal rooms were in buildings separate from that in which this laboratory is situated. Cotton rats, hamsters, and guinea pigs were kept in this laboratory either in metal cages or in individual glass jars with wire screen covers. At times they were kept in separate animal rooms which housed no other species; at other times in animal rooms in which other species were present. In certain experiments animals were strictly isolated during the entire period of observation in special metal cubicles (6).[1]

Individual animals were identified by means of serially numbered ear tags in order that multiple serum specimens obtained at different times from the same animal might be compared directly.

Serum.—As soon as practicable after arrival in this laboratory a specimen of serum was obtained with aseptic precautions from each animal. In the great majority of instances the initial serum was obtained within the first week following the animal's arrival in the laboratory. In many cases additional specimens of serum were obtained at later times, after periods of observation or following various experimental procedures. All specimens of serum were stored under sterile conditions without preservative at 4°C.

Virus.—Pneumonia virus of mice, strain 15 (1), was used throughout this study. Suspensions of mouse lungs infected with the virus were prepared, stored, and titered in a manner identical to that described in the preceding paper (2). For convenience this virus will be referred to hereafter as PVM.

Neutralization Test.—The technique used in neutralization tests throughout this study was identical to that described in the preceding paper (2). Serum specimens were inactivated

[1] The special isolation facilities were made available through the kindness of Dr. G. K. Hirst, International Health Division of The Rockefeller Foundation, New York.

at 56°C. for 30 minutes and serial fivefold dilutions in saline were prepared. The virus suspension was diluted to the desired degree with broth containing 20 per cent normal horse serum, previously inactivated at 56°C. for 30 minutes. Equal quantities of each serum dilution and the diluted virus suspension were thoroughly mixed and approximately 30 to 60 minutes later a group of three mice was inoculated with each serum-virus mixture. Albino Swiss mice were used, and each mouse received 0.05 cc. intranasally under light ether anesthesia. The inoculated mice were observed daily for 11 to 12 days; the lungs of those which died were examined for the presence and the degree of pulmonary consolidation; those which survived were killed, and their lungs examined similarly for pulmonary lesions.

Since the titer of the virus suspension was determined prior to its use in almost all tests, it was possible to test each serum against a relatively constant quantity of virus. Additional virus titrations were carried out simultaneously with each neutralization test to assure that the desired amount of virus had actually been employed. In all tests between 10 and 100 (a mean of 30) 50 per cent maximum score doses of virus were used.

Calculation of End Points.—The 50 per cent end point calculation method of Reed and Muench (7) was used for the determination of all titers, both virus and serum. Throughout this study the 50 per cent maximum score end point described previously (8) was used, and will be referred to hereafter as M.S. 50. That this end point was sufficiently reproducible to be useful in studies on PVM and antibodies against it was shown in the preceding paper (2).

Significance of Neutralization Titers.—For the purposes of this study it was necessary to define those serum neutralization titer levels which could be considered to have a high probability of being significant. On the basis of the studies on PVM presented in the preceding paper (2), this can be accomplished in two different ways.

Firstly, by use of the equation which was shown to express the linear relationship between neutralization titer and amount of virus used (2) it is possible to calculate the neutralizing *capacities* (8) of sera which show various neutralization titers against a known amount of virus. When this was done it was found that sera which showed neutralization titers of from 1:2 to 1:9 against log 1.5 M.S. 50 doses of virus had, on the average, neutralizing capacities no higher than log 1.9. However, sera which showed, under similar conditions, neutralization titers of from 1:10 to 1:50, and from 1:51 to 1:250 or more, were found to have neutralizing capacities of at least log 3.4 and log 4.4, respectively. It is evident that sera with neutralization titers of from 1:2 to 1:9, on the average, were capable of neutralizing no more than 80 M.S. 50 doses of PVM, whereas sera with titers of from 1:10 to 1:50 and from 1:51 to 1:250 or more, were capable of neutralizing as much as 2,500 and 25,000 M.S. 50 doses of virus, respectively.

Secondly, by use of the standard deviation (0.221 log units) of the distribution of serum dilution end points determined for neutralization tests with PVM (2), it is possible to calculate the significance of differences between end points. When this was done it was found that, with sera which showed neutralization titers of from 1:2 to 1:9, the probability was on the average no greater than 0.83 that the results were significant. On the other hand, with sera which showed, under similar conditions, neutralization titers of from 1:10 to 1:50, and from 1:51 to 1:250 or more, the probabilities were at least 0.999 and 0.9999, respectively, that the results were significant.

On the basis of the results obtained by both methods of anaylsis given above, it seemed obvious that, under the conditions of these experiments, neutralization titers of from 1:2 to 1:9 were of doubtful significance, whereas titers of from 1:10 to 1:50, and from 1:51 to 1:250 or more were highly significant. Consequently, throughout this paper, those sera which yielded titers of from 1:2 to 1:9 will be grouped together with sera which gave zero titers, and it will be considered that with none of the sera in this group was unequivocal evidence of the presence of neutralizing antibodies against PVM obtained. Conversely, it will be assumed that titers of from 1:10 to 1:50, and more than 1:50 definitely indicated the presence of such antibodies.

EXPERIMENTAL

Incidence of Antibodies in Serum of Normal Animals.—Serum specimens from apparently healthy animals which had not been subjected to any experimental procedure, as well as serum specimens from normal adult human beings were tested against PVM by the neutralization technique described above. The 50 per cent maximum score end point (M.S. 50) was determined for each serum. The results of these tests are presented in Table I. The significance of the neutralization titers in the various titer groups shown was discussed above. Throughout this paper neutralization titers of 1:9 or less are considered to be not significant.

TABLE I

Results of Neutralization Tests with PVM and Serum of Normal Animals and Normal Human Beings

Normal serum		No. of sera showing indicated neutralization titers M.S. 50			Per cent of sera with neutralization titers of 1:10 or higher
Species	No. tested	0–1:9	1:10–1:50	>1:50	
Chicken	24	24	0	0	0.0
Mongoose	10	10	0	0	0.0
Rabbit	210	182	24	4	13.3
Cotton rat	1458	1129	113	216	22.6
Monkey	106	81	25	0	23.6
Human	165	118	37	10	27.4
Chimpanzee	6	4	2	0	33.3
Hamster	580	298	24	258	48.6
Guinea pig	60	20	12	28	66.7
Mouse	14*	2	5	7	85.7
Total	2633	1868	242	523	29.0

* Each of these specimens was a pool obtained from six or more mice.

It will be seen that none of the sera from normal chickens, the only avian species tested, was capable of neutralizing PVM. It seems of interest to emphasize that all attempts to demonstrate multiplication or the presence of this virus in chick embryos following inoculation have been uniformly unsuccessful. Moreover, attempts to stimulate the production of neutralizing antibodies by the injection of large amounts of active PVM in chickens have been equally unsuccessful. The available evidence suggests that chickens not only do not harbor a latent agent similar to this virus, but may possess the capacity to destroy PVM, since in them it was not antigenic.

It will be noted that of the nine mammalian species included in this study, a certain proportion of the serum specimens from normal human beings and normal animals of each species, excepting only the mongoose, were capable of

neutralizing PVM. Considering the small number of sera from normal mongoose which were available, these results cannot be given much weight, particularly since, as will be shown below, other evidence has been obtained which suggests that the mongoose also may harbor a latent agent closely related to PVM.

In Table I the various species are arranged according to the frequency with which neutralizing antibodies against PVM were demonstrated in sera from normal members of each species. It will be observed that among the animals tested, normal rabbits showed a lower incidence (13.3 per cent) of antibodies against PVM than any other mammalian species from which an adequate number of sera were available. As was to be anticipated, normal mice, from the lungs of which PVM was originally obtained, showed a higher incidence (85.7 per cent) of neutralizing antibodies in their pooled sera than any other species tested. The frequency with which normal guinea pigs, hamsters, and cotton rats showed relatively high titers of neutralizing antibodies against PVM was very surprising, and in the case of the latter two species the significance of these findings is greatly enhanced by the large numbers of sera tested.

The finding that 27.4 per cent of sera from normal human beings tested during this study were capable of neutralizing PVM agrees very closely with results obtained some years ago, when it was found that 32.8 per cent of similar human sera neutralized PVM (1).

In a large number of instances the same sera from various species were tested twice in different neutralization tests. Almost without exception sera which did not show significant neutralization titers in the first test also failed to show such titers in the second test. Conversely, sera which gave neutralization titers of 1:10 or more in the first test, in almost every instance gave similar titers in the second test.

These results indicate clearly that a varying proportion of sera from a wide variety of healthy and apparently normal mammalian species, including human beings, were capable of neutralizing PVM. The available evidence indicates that, under the conditions of these experiments, neutralization of this virus by a given serum resulted from the presence in the serum of specific antibodies against the virus. It seems probable that the presence of such antibodies indicates a previous experience either with PVM or an agent antigenically similar to it. Therefore the observed results suggest either that this virus itself is widely distributed among mammalian species, or that other agents closely related to it are so distributed.

Seasonal Variations in Incidence of Antibodies

Normal cotton rats and normal hamsters, approximately 5 weeks of age, were received every 2 weeks during each month from January, 1943, through February, 1945. Serum specimens were obtained from many of these animals within

a few days after their arrival in this laboratory, and were tested, by the neutralization technique described above, against PVM. From June, 1942, through January, 1945, groups of normal rabbits were also received, and early serum specimens obtained from them were tested similarly.

TABLE II

Results of Neutralization Tests with PVM and Serum of Young, Normal Cotton Rats

Date		No. cotton rats	No. of sera showing indicated neutralization titers M.S. 50		Per cent with neutralization titers of 1:10 or higher
Year	Month		0-1:9	1:10 or higher	
1943	1	77	57	20	26.0
	2	38	25	13	34.2
	3	58	47	11	19.0
	4	100	78	22	22.0
	5	56	40	16	28.6
	6	97	61	36	37.1
	9	44	43	1	2.3
	10	89	89	0	0.0
	11	173	173	0	0.0
	12	104	104	0	0.0
1944	1	154	150	4	2.6
	3	83	27	56	67.4
	5	34	8	26	76.4
	6	19	7	12	63.2
	8	24	10	14	58.3
	9	24	12	12	50.0
	10	80	60	20	25.0
	11	67	57	10	14.9
	12	43	39	4	9.3
1945	1	72	40	22	30.6
	2	22	2	20	90.9
Total.....		1458	1129	329	22.6

The results of neutralization tests with serum of young, normal cotton rats and PVM are shown in Table II. For the purposes of this analysis, animals were grouped together according to the month in which they were received in this laboratory. Each specimen of serum tested was the first obtained from a single young cotton rat, and only this one serum specimen from each cotton rat was used in this particular study.

It will be noted that during each month from January through June in 1943, neutralizing antibodies were demonstrated in the serum of approximately one in every four normal cotton rats tested; the mean incidence of such antibodies was 27.7 per cent. From September, 1943,

through January, 1944, neutralizing antibodies were demonstrable in the serum of only one in every 110 normal cotton rats tested, and during a period of 3 consecutive months, none of a large number of animals showed such antibodies. Throughout the whole period, the mean incidence was 0.9 per cent.

From March through September in 1944, the incidence of neutralizing antibodies in serum of normal cotton rats again increased; approximately two of every three animals tested showed such antibodies; the mean incidence was 65.2 per cent. From October through December, 1944, the frequency with which neutralizing antibodies were demonstrated again sharply

TABLE III

Results of Neutralization Tests with PVM and Serum of Young, Normal Hamsters

Date		No. hamsters	No. of sera showing indicated neutralization titers M. S. 50		Per cent with neutralization titers of 1:10 or higher
Year	Month		0–1:9	1:10 or higher	
1943	1	16	15	1	6.3
	3	20	16	4	20.0
	6	25	24	1	4.0
	9	50	50	0	0.0
	10	47	47	0	0.0
	11	46	46	0	0.0
	12	21	21	0	0.0
1944	1	54	54	0	0.0
	3	21	0	21	100.0
	5	47	9	38	80.0
	6	10	1	9	90.0
	8	18	0	18	100.0
	9	24	0	24	100.0
	10	44	12	32	72.7
	11	73	0	73	100.0
	12	21	0	21	100.0
1945	1	23	3	20	86.9
	2	20	0	20	100.0
Total		580	298	282	48.6

declined; the mean incidence was 17.9 per cent. During the first two months of 1945, a marked rise in incidence of neutralizing antibodies in serum of normal cotton rats again occurred, and during February, nine out of every ten animals tested showed such antibodies, the highest incidence observed during the entire 26 month period.

The results of neutralization tests with serum of young, normal hamsters and PVM are presented in Table III. In this analysis, also, animals were grouped together according to the month in which they were received in the laboratory, and the significance of various neutralization titers was considered to be identical to that described above.

It will be observed that from January through June in 1943, neutralizing antibodies were demonstrated in the serum of approximately one in every ten normal hamsters tested. However, during a period of 5 months from September, 1943, through January, 1944, none of a large number of animals showed such antibodies From March, 1944, through February, 1945, neutralizing antibodies were encountered in the serum of approximately nine in every ten normal hamsters tested; the mean incidence was 91.7 per cent.

TABLE IV

Results of Neutralization Tests with PVM and Serum of Normal Rabbits

Date		No. rabbits	No. of sera showing indicated neutralization titers M. S. 50		Per cent with neutralization titers of 1:10 or higher
Year	Month		0-1:9	1:10 or higher	
1942	6	6	4	2	33.3
	7	2	2	0	0.0
	8	6	6	0	0.0
	9	6	6	0	0.0
	10	7	7	0	0.0
	11	12	12	0	0.0
	12	20	18	2	10.0
1943	1	19	18	1	5.3
	5	33	29	4	12.1
	6	10	9	1	10.0
	9	3	3	0	0.0
	10	34	30	4	11.8
1944	1	10	8	2	20.0
	8	8	6	2	25.0
	11	10	6	4	40.0
1945	1	24	18	6	25.0
Total.....		210	182	28	13.3

The results of neutralization tests with serum of normal rabbits and PVM are shown in Table IV. Again in this analysis animals were grouped together according to the month in which they were received.

It will be seen that during 1942, neutralizing antibodies were demonstrated in the serum of normal rabbits in June and December, but that none of the animals bled during the period from July through November showed such antibodies. Throughout the last 7 months in 1942, only one in every fifteen normal rabbits gave evidence of possessing antibodies capable of neutralizing PVM. During 1943, the serum of one in every ten normal rabbits contained neutralizing antibodies; the mean incidence was 10.1 per cent. During 1944, and the first month in 1945, approximately one in every four normal rabbits showed such antibodies; the mean incidence was 26.9 per cent.

It is of importance to emphasize that the observed differences in the frequencies of neutralizing antibodies were not related to differences in the quantity of virus used in different neutralization tests. Analysis of all data pertinent to this possibility, as well as repeated neutralization tests with many specimens of serum, revealed that the differences noted were not attributable to slight and unavoidable variations in experimental conditions, but to actual differences in the neutralizing capacities of the various serum specimens tested.

The frequency with which neutralizing antibodies against PVM were found in the serum of normal animals belonging to three species is presented graphi-

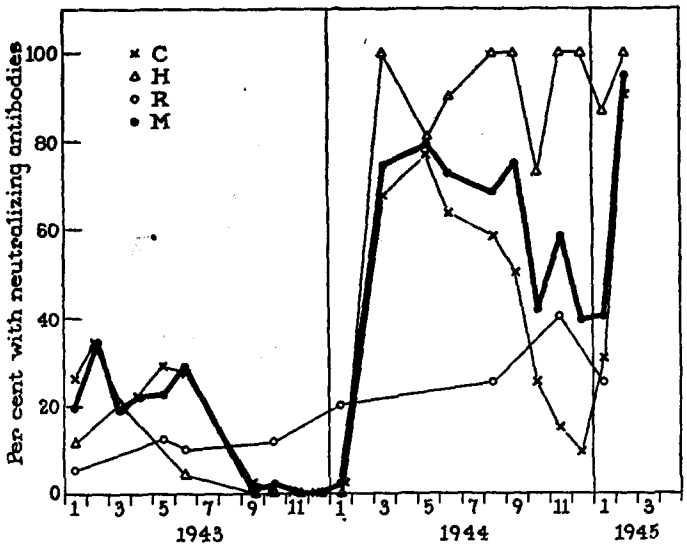

FIG. 1. The incidence of neutralizing antibodies against PVM in the serum of normal cotton rats (C), hamsters (H), and rabbits (R) in relation to various seasons. (M) = mean incidence for the three species.

cally in Fig. 1. The frequencies were taken from the data shown in Tables II, III, and IV. The percentage incidence of such antibodies in each species, as well as the mean for the three species, was plotted against the time when the animals were received. It will be observed that the incidence of neutralizing antibodies in each species was greater during 1944 than during 1943; and in the case of cotton rats and hamsters, the incidence was much greater. It will be noted that the frequency with which normal rabbits showed such antibodies did not vary widely in either year, whereas in both normal cotton rats and hamsters enormous variations in incidence were encountered. It appears evident from the antibody-frequency time curve developed for cotton rats, that the incidence of neutralizing antibodies in this species was correlated with the season.

Cotton rats born during the late winter and spring months in either year showed such antibodies upon arrival in the laboratory much more commonly than did cotton rats born during the summer and fall months. During 1943, a closely similar seasonal variation in incidence of antibodies was observed with normal hamsters, but in the following year no corresponding reduction in frequency occurred with this species during the last half of the year. Because of the relatively large number of cotton rats tested, at each interval, it seems probable that the results obtained with this species gave a more reliable indication of the seasonal variations in incidence of neutralizing antibodies than did those obtained with either hamsters or rabbits.

Effect of Non-Specific Stimulation upon the Development of Antibodies

Attempts were made to determine what effect the injection of various materials which did not contain PVM would have upon the development of neutralizing antibodies against this virus in normal animals.

At intervals during the period from July, 1942, through February, 1945, groups of normal cotton rats and hamsters under ether anesthesia, were given intranasal injections of either sterile broth, normal chick embryo suspensions, specimens obtained from patients with primary atypical pneumonia, or embryo passage material derived from such specimens. Simultaneously, other groups of normal cotton rats and hamsters which received no injection, were held as controls. From July, 1942, through December, 1944, groups of normal rabbits were given intraperitoneal or intravenous injections of either normal chick embryo suspensions, heat-killed suspensions of non-hemolytic streptococci, specimens from patients with primary atypical pneumonia, or embryo passage material derived from such specimens. Other normal rabbits which received no injection, served as controls.

Serum was obtained from each animal shortly before the injection was given, and again approximately 3 weeks following the injection. In the case of cotton rats and hamsters, all bleedings were carried out under ether anesthesia, consequently each animal in a control group was anesthetized twice, once for each serum specimen, whereas each animal in a group which received injections was anesthetized thrice, once for each serum specimen, as well as once for the injection. Rabbits were not anesthetized at any time. Neutralization tests with PVM were performed with each serum according to the technique described above.

The results of neutralization tests with PVM and serum of cotton rats obtained before, and 3 weeks following intranasal injection, are shown in Table V. Only those animals which showed no evidence of neutralizing antibodies in serum obtained before injection, were included in this analysis.

It will be observed that among cotton rats which received sterile broth intranasally, the frequency with which neutralizing antibodies developed during the observation period was almost identical with that found among animals which received no injection. The mean frequency with which antibodies developed in both groups was 20.8 per cent. It will also be noted that among cotton rats which received normal chick embryo suspensions intranasally, the frequency with which neutralizing antibodies developed was nearly identical with that found among animals which received specimens or embryo passage materials derived from patients with primary atypical pneumonia. The mean frequency with which such antibodies

developed in these latter groups was 50.8 per cent. It is apparent that in cotton rats which received chick embryo materials or materials obtained from human beings, neutralizing antibodies against PVM developed approximately 2.4 times more frequently than in similar animals which either received sterile broth or were not injected. Statistical analysis revealed that this difference was highly significant, and would be expected to occur by chance only once in over 100,000 times.

The results of neutralization tests with PVM and serum of hamsters obtained before, and 3 weeks following intranasal injection, are shown in Table VI. As

TABLE V

Results of Neutralization Tests with PVM and Serum of Cotton Rats Which Received Intranasal Injections

Intranasal injection: Material	No. cotton rats	No. of sera showing indicated neutralization titers M. S. 50			Per cent developing neutralization titers of 1:10 or higher
		Before injection	3 wks. after injection		
		0–1:9	0–1:9	1:10 or higher	
None	44	44	34	10	22.7
Broth	52	52	42	10	19.2
Total	96	96	76	20	20.8
Normal embryo	81	81	40	41	50.6
PAP* materials	96	96	47	49	51.0
Total	177	177	87	90	50.8

* PAP materials include specimens and chick embryo passage materials derived from patients with primary atypical pneumonia.

in the preceding analysis, only those animals which showed no evidence of neutralizing antibodies before injection, were included.

It will be observed that, among hamsters which either received sterile broth or were not injected, only two of a total of twenty-nine animals developed neutralizing antibodies against PVM. On the other hand, among hamsters which received normal chick embryo suspensions or materials derived from patients with primary atypical pneumonia, nine of a total of thirty-six animals developed such antibodies. It is evident that hamsters given the latter materials produced antibodies against this virus approximately 3.6 times more frequently than animals in the first two groups. This difference is similar to that observed under identical experimental conditions with cotton rats, and statistical analysis revealed that it was moderately significant, since it would be expected to occur by chance alone only once in nineteen times.

The results of neutralization tests with PVM and serum of rabbits obtained before, and 3 or more weeks following either intraperitoneal or intravenous injections, are shown in Table VII. As in the previous experiments, animals

were included in this analysis only if serum obtained before injection showed no evidence of neutralizing antibodies.

TABLE VI

Results of Neutralization Tests with PVM and Serum of Hamsters Which Received Intranasal Injections

Intranasal injection	No. hamsters	No. of sera showing indicated neutralization titers M. S. 50			Per cent developing neutralization titers of 1:10 or higher
Material		Before injection	3 wks. after injection		
		0–1:9	0–1:9	1:10 or higher	
None	10	10	10	0	0.0
Broth	19	19	17	2	10.5
Total	29	29	27	2	6.9
Normal embryo	15	15	12	3	20.0
PAP materials	21	21	15	6	28.6
Total	36	36	27	9	25.0

TABLE VII

Results of Neutralization Tests with PVM and Serum of Rabbits Which Received Intraperitoneal or Intravenous Injections

Material injected	No. rabbits	No. of sera showing indicated neutralization titers M. S. 50			Per cent developing neutralization titers of 1:10 or higher
		Before injection	3 wks. or more after injection		
		0:1:9	0–1:9	1:10 or higher	
None	14	14	14	0	0.0
Normal embryo	28	28	25	3	10.7
PAP materials	63	63	50	13	20.6
Non-hemolytic streptococci	27	27	19	8	29.6
Total	118	118	94	24	20.3

It will be noted that among fourteen rabbits which received no injection, none developed neutralizing antibodies. Among rabbits which received either suspensions of normal embryos, specimens of embryo passage material derived from patients with primary atypical pneumonia, or suspensions of heat-killed non-hemolytic streptococci, from 10.7 to 29.6 per cent, a mean of 20.3 per cent, developed antibodies against PVM. Statistical analysis showed that the ob-

served difference between the injected and the control animals was of moderate significance, since it would be expected to occur by chance alone only once in seventeen times.

Injections similar to those described above were carried out with the wild mongoose in Puerto Rico by the Commission on Acute Respiratory Diseases, Fort Bragg, North Carolina, which kindly supplied specimens of serum from these animals. The results of neutralization tests against PVM with these mongoose sera indicated that following intranasal injection with either broth or suspensions of mongoose lung, none of twelve animals developed significant antibody titers. However, following intranasal injection of specimens obtained

TABLE VIII

Results of Neutralization Tests with PVM and Serum of Cotton Rats, Hamsters, and Rabbits before and after Non-Specific Stimulation

Material injected	No. animals	No. of sera showing indicated neutralization titers M.S. 50			Per cent developing neutralization titers of 1:10 or higher
		Before	3 wks. after injection		
		0–1:9	0–1:9	1:10 or higher	
None	68	68	58	10	14.2
Broth	71	71	59	12	16.9
Total	139	139	117	22	15.8
Normal embryo	124	124	77	37	37.9
PAP materials	180	180	112	68	37.8
Total	304	304	189	115	37.8

from patients with primary atypical pneumonia, three of ten mongoose developed neutralization titers against this virus of 1:10 or more.

The results of neutralization tests with PVM and serum of cotton rats, hamsters, and rabbits, obtained before and 3 or more weeks following non-specific stimulation by the injection of various materials are presented together in Table VIII. The results obtained with each of these species individually are shown in Tables V, VI, and VII, respectively.

It will be seen that when the results with the three species tested were considered together, there was very little difference in the frequency with which antibodies against PVM developed in animals following the injection of sterile broth (16.9 per cent) as compared with uninjected controls (14.2 per cent). Similarly, the frequency of the development of such antibodies was almost identical in animals which were given normal embryo suspensions (37.9 per cent), as compared with other animals which received specimens or embryo passage materials derived from patients with primary atypical pneumonia (37.8 per cent). It is evident that neutralizing

antibodies developed, on the average, 2.4 times more commonly in animals which received the latter materials than in those which either were not injected or received sterile broth. This difference is highly significant, since the probability that it would occur by chance in groups of this size was found to be less than 1 in 160,000.

It should be emphasized that results closely similar to those shown in Tables V and VI were obtained both in cotton rats and in hamsters which, during the entire period of observation, were kept in strict isolation in relatively air-tight metal cubicles (6). Consequently, it seems very unlikely that the development of neutralizing antibodies against PVM ,which was observed in these animals, is to be explained on the basis of accidental contact infection with the virus in the laboratory.

These results indicate that certain types of non-specific stimulation, *e.g.* the injection of native materials derived from other species, induced, in the three species tested, development of specific antibodies against a latent virus, more commonly than occurred in the absence of such stimulation, or when autoclaved broth was given. Moreover, these findings raise the possibility that the effective non-specific stimuli caused an imbalance in an equilibrium between host and latent agent which favored the latter, and resulted in a specific antibody response against the virus. It seems probable that this response was a direct result of inapparent infection by the latent virus.

Relation between Incidence of Antibodies in Normal Animals and Effect of Non-Specific Stimulation

As was shown above, the incidence of neutralizing antibodies against PVM in serum of normal cotton rats, varied markedly with the season, and was lowest during the summer and fall months. It was also shown that following the intranasal injection of certain native foreign materials, *e.g.* normal chick embryo syspensions, cotton rats developed neutralizing antibodies against PVM more often than uninjected control animals or those given sterile broth. It appeared of interest to determine whether the frequency with which specific neutralizing antibodies were produced following such non-specific stimulation was related to the incidence of neutralizing antibodies in the serum of normal animals.

The results of neutralization tests with PVM and serum of normal cotton rats were compared with the results of similar tests with serum obtained from cotton rats before and 3 weeks after intranasal injection of materials which did not contain PVM. Since normal animals showed neutralizing antibodies with considerable frequency during the late winter and spring months of both 1943 and 1944, whereas such antibodies were not found in similar animals, with but rare exceptions, during the fall and early winter of 1943–44, the results of tests in cotton rats with non-infectious materials carried out during each of these periods were analyzed. The materials given intranasally in these tests included sterile broth and normal embryo suspensions, as well as specimens or embryo passage materials derived from patients with primary atypical pneumonia.

The results of these analyses are presented in Table IX. It will be noted that during the period from January through June in 1943 the average incidence of neutralizing antibodies in serum of normal cotton rats was 27.7 per cent; from November, 1943, through January, 1944, the mean incidence was only 1.0 per cent; while from March through September in 1944 it was 65.2 per cent. It will also be seen that during these same periods, the mean frequencies with which such antibodies developed following intranasal injection of foreign materials in cotton rats, which initially did not possess antibodies, were 57.7 per cent, 2.0 per cent, and 33.3 per cent, respectively. The differences observed between either the incidence of antibodies in serum of normal animals, or the frequency of the development of antibodies following injection of foreign materials, during the fall and early winter months of 1943–44 and similar data for the spring and

TABLE IX

Results of Neutralization Tests with PVM and Serum of Cotton Rats Which Received Intranasal Injections during Different Seasons

Season	Injected cotton rats					Normal cotton rats		Total cotton rats showing evidence of latent infection with PVM
	No. tested	No. of sera showing indicated neutralization titers M.S. 50			Per cent developing neutralization titers of 1:10 or higher	No. tested	Per cent with neutralization titers of 1:10 or higher	
		Before injection	3 wks. after injection					
		0–1:9	0–1:9	1:10 or higher				
								Per cent
Jan.–June 1943	111	111	47	64	57.7	426	27.7	69.4
Nov., 1943 to Jan., 1944	100	100	98	2	2.0	431	1.0	3.0
Mar.–Sept., 1944	27	27	18	9	33.3	184	65.2	76.8

summer months of both 1943 and 1944 were found to be highly significant. The probability that any of these differences occurred by chance alone is less than 1 in 1,000,000.

These results suggest that there was a direct relationship between the incidence of neutralizing antibodies against PVM in serum of normal animals, and the frequency with which antibodies against this virus developed, following non-specific stimulation, since both varied widely and in a similar manner throughout three successive seasons.

If the number of animals which showed the presence of neutralizing antibodies upon arrival in the laboratory was subtracted from the total number tested, the remaining animals, which did not possess such antibodies, could be divided into three separate groups: (1) those animals which went on to develop neutralizing antibodies in the absence of any purposefully applied stimulus; (2) those animals which developed such antibodies when an appropriate non-specific stimulus was given; and (3) those animals which did not develop such antibodies even when an effective non-specific stimulus was given. On this basis, it was possible to calculate the per-

centage of all the animals in a given group adequately tested, which yielded any evidence of latent infection by this virus. Such calculations were carried out for the large groups of animals studied during the three successive seasons, and these results are also shown in Table IX. It will be observed that during the fall and early winter season of 1943–44, only 3.0 per cent of all the animals studied showed any evidence of latent infection by PVM, whereas during the spring and summer seasons of 1943 and 1944, 69.4 per cent and 76.8 per cent, respectively, yielded such evidence.

These results suggest that during both the spring and summer seasons under consideration, this virus was very frequently harbored by normal cotton rats, whereas during the intervening fall and early winter season it was only very rarely harbored by similar animals obtained from the same source.

Effect of Inoculation with PVM in Animals Which Showed No Evidence of Latent Infection

As was shown above, certain animals not only did not possess neutralizing antibodies against PVM, but also failed to develop such antibodies following appropriate non-specific stimulation. Moreover, it was shown that the percentage of such animals was much greater during the fall and early winter season than during the spring and summer seasons. Inasmuch as these animals showed no evidence of latent infection with PVM, it was of interest to determine whether they were actually susceptible to infection with the virus and would develop antibodies against it.

In January, 1944, when the incidence of neutralizing antibodies in the serum of normal cotton rats was found to be very low, *e.g.* 2.6 per cent, each of a group of cotton rats was given intranasally either a specimen or embryo passage material derived from patients with primary atypical pneumonia. In a number of instances, the materials which were injected were identical with those which had been used during 1942 and 1943 for the injection of cotton rats and rabbits in attempts to recover a virus from patients with primary atypical pneumonia (9). 3 weeks after this injection, serum was obtained from each animal. After an additional period of 3 weeks, half of the group were given intranasally 0.25 cc. of a 10^{-2} dilution of a suspension of mouse lungs infected with PVM, and the other half of the group were given 0.25 cc. of sterile broth intranasally. 3 weeks after these latter injections, serum was again obtained from each animal. The presence or absence of nautralizing antibodies against PVM in each serum was determined by the technique described above.

The results of neutralization tests with PVM and multiple serum specimens from cotton rats which received two successive intranasal injections are shown in Table X. It will be noted that following the first intranasal injection with materials derived from patients with primary atypical pneumonia, none of the animals developed neutralizing antibodies. It will also be seen that those animals which were given sterile broth 6 weeks after the first injection, failed to develop such antibodies, whereas those which received PVM at the same time produced antibodies against the virus without exception, and all developed neutralization titers greater than 1:50.

These results indicate clearly that cotton rats which failed to show evidence of latent infection with PVM, even after receiving appropriate non-specific stimuli, were none the less susceptible to induced infection with the virus and capable of developing specific antibodies against it. Inasmuch as the animals which received PVM were not isolated from those which were given sterile broth, but instead were kept individually in adjacent glass jars supplied with wire screen tops, the results also indicate that PVM was not transmitted from one cotton rat to another, even though they were in close proximity.

It is of importance to emphasize that when tested in January, 1944, none of the specimens obtained from patients with primary atypical pneumonia, and none of the embryo passage materials derived from them stimulated the production of neutralizing antibodies against PVM following intranasal injection in cotton rats. It is apparent that these results are not in agreement with the results of similar tests on the same specimens carried out in this laboratory dur-

TABLE X

Results of Neutralization Tests with PVM and Multiple Serum Specimens from Cotton Rats Which Received Two Successive Intranasal Injections

1st intranasal injection	No. cotton rats	No. of sera showing indicated neutralization titers M.S. 50			Interval	2nd intranasal injection	No. of sera showing indicated neutralization titers M. S. 50	
Material		Before injection	3 wks. after 1st injection			Material	3 wks. after 2nd injection	
		0–1:9	0–1:9	1:10			0–1:9	>1:50
					wks.			
PAP materials.....	21	21	21	0	3	PVM.....	0	21
PAP materials.....	22	22	22	0	3	Broth.....	22	0

ing 1942 and 1943 (9). The explanation for the different results obtained seems obvious, in the light of the findings presented in this paper, particularly the experimental data shown in Tables V, VII, VIII, and IX.

The available evidence indicates clearly that specimens and embryo passage material derived from patients with primary atypical pneumonia did not stimulate the production of neutralizing antibodies against PVM following their injection in either cotton rats, hamsters, or rabbits any more frequently than did normal embryo suspensions. Consequently, it seems probable that both types of material acted as non-specific stimuli which served only as evokers of a preexisting latent virus infection in each of these species.

Effect of Inoculation with PVM in Animals Which Possessed Antibodies

The results of previous experiments (1) indicated that PVM was pathogenic for mice, but was non-pathogenic for a number of different mammalian species,

including the hamster. Further study has revealed that this virus is, in fact, pathogenic for certain species other than the mouse. As will be shown below, evidence has been obtained which indicates that in either hamsters or cotton rats which do not possess neutralizing antibodies against it, PVM is capable of inducing extensive pneumonia which is sometimes fatal.

Normal cotton rats and hamsters, as well as similar animals which had been subjected to various experimental procedures, were inoculated intranasally with PVM. Suspensions of consolidated lungs obtained from either cotton rats or hamsters purposely infected with PVM were used, and each animal was given 0.25 cc. under light ether anesthesia. From 7 to 9 days following inoculation the animals were killed, and their lungs examined. The extent of the total lung tissue which showed consolidation was estimated and recorded in a manner identical to that previously described for similar lesions in mice (1). Serum obtained from each animal shortly before inoculation was tested for neutralizing antibodies against PVM by the technique described above.

TABLE XI

Results of Inoculation with PVM in Cotton Rats and Hamsters in Relation to Neutralizing Antibody Levels against the Virus

Neutralization titers of serum M.S. 50	Species	No. animals tested	Intranasal inoculation	No. of animals showing pulmonary lesions of indicated extent							Per cent developing penumonia
				0	±	+	++	+++	++++	D++++	
0–1:9.....	Cotton rat	92	PVM	9	21	20	24	12	4	2	90.2
0–1:9.....	Hamster	73	PVM	0	2	1	6	13	33	18	100.0
1:10 or higher.	Cotton rat	33	PVM	33	0	0	0	0	0	0	0.0
1:10 or higher.	Hamster	29	PVM	29	0	0	0	0	0	0	0.0

The results of these tests are shown in Table XI. It will be noted that the inoculation of PVM in cotton rats and hamsters, which prior to inoculation did not show significant serum antibody titers against the virus, was followed by the development of pneumonia in 90.2 per cent and 100.0 per cent of animals, respectively. Approximately 96 per cent of the hamsters in this group showed extensive pulmonary consolidation, involving one-half or more of the total lung tissue, and almost 25 per cent developed fatal pneumonia. Among cotton rats which did not possess neutralizing antibodies, approximately 46 per cent showed similarly extensive consolidation, and 2 per cent developed fatal pneumonia. It will be seen also that in both cotton rats and hamsters, which showed neutralizing antibody titers of 1:10 or more against PVM, the inoculation of the virus was not followed by the development of pulmonary lesions in a single instance. It is of importance to emphasize that the presence or absence in the serum of a significant antibody titer was the sole criterion used for the separation of the animals in each species into two groups. Consequently, each group contained normal animals which had not been subjected

to any experimental procedure, as well as animals which previously had received intranasal injections of various foreign materials. It was found that there was no correlation between the previous experimental experience of the animals in each group and the result of direct tests for susceptibility to infection with PVM.

These results indicate that both cotton rats and hamsters which did not possess demonstrable circulating antibodies against PVM, almost without exception, were susceptible to manifest pulmonary infection by the virus, whereas similar animals which showed significant neutralizing antibody titers were immune.

DISCUSSION

The results of the experiments described in this paper indicate that in each of nine mammalian species a varying proportion of individuals gave evidence of latent infection with either PVM or a virus antigenically similar to it. That these latent infections resulted from contact with mice, many of which carry this virus, seems very unlikely. Most of the animals studied were raised and housed under conditions which almost continuously excluded the possibility of contact with mice. Moreover, numerous tests were carried out with animals which were isolated in the laboratory either in separate animal rooms, or in special metal cubicles, and had, so far as could be determined, no opportunity for contact with mice. As is evident from the results obtained, a surprisingly high percentage of animals on arrival in the laboratory showed the presence of circulating antibodies against PVM; and so, too, did normal human beings. That the presence of neutralizing antibodies in the serum was of significance, and that such antibodies were indicative of a specifically altered immunological status is evident from the finding that both cotton rats and hamsters which possessed circulating antibodies were solidly immune to infection with PVM, whereas those without demonstrable antibodies were susceptible to manifest pulmonary infection with this virus.

The present immunological evidence suggests that either this virus or one closely related to it in antigenic structure is harbored frequently by numerous mammalian species; that latent infection with PVM is not confined to mice, or in fact to rodents, but occurs commonly among monkeys, and even among human beings. It seems evident that animals which harbor the virus derive it from other animals in which the agent is present. Whether transmission from one animal to another occurs directly or indirectly through some intermediary host, is not yet known. However, since PVM has not been obtained from any tissue other than lung, and all available evidence indicates that the virus is strictly pneumotropic, it seems probable that direct transmission could occur, and that the portal of entry might be the respiratory tract. On the other hand, numerous direct attempts to demonstrate transmission of PVM

by contact have regularly failed, either between animals of the same species or between animals of different species. It seems possible, however, that the failures to demonstrate contact transmission may be more apparent than real, and may have resulted from the inability of the animals tested to transfer quantities of the virus sufficient to induce detectable pulmonary disease or alterations in the immunological status of the potential recipient. Inasmuch as this virus appears to be widely disseminated among many different mammalian species, it seems evident that it is readily transmitted from one animal to another, as well as from one species to another under natural conditions. This is the more surprising when it is recalled that PVM is a very labile virus which becomes inactivated as an infectious agent in a relatively few hours at room temperature, and consequently it seems likely that the virus could not retain the property of infectiousness for more than a very short time in the usual environments of either man or animals.

Marked seasonal differences in the incidence of numerous infectious diseases, particularly virus infections, are of course well known, although the factors which are responsible for such differences, in most instances, are either vaguely or not at all understood. It has been shown that the incidence of latent infection with PVM also has striking seasonal variations, especially among cotton rats. Because direct means for the detection of very small amounts of PVM are not yet available, it was not possible to determine whether the virus was actually harbored by animals which failed to manifest any evidence of latent infection with it. If the virus was in fact harbored by such animals, it seems probable that it was present either in a much smaller quantity, or in a qualitatively different state as compared to its status in those animals which gave evidence of latent infection. However, since none of the animals tested in one season, whereas almost all of those tested in another showed evidence of latent infection with PVM, it seems reasonable to suppose that the virus was harbored much less commonly during the season first mentioned.

When a susceptible host harbors or carries a potentially infectious agent, and no detectable reaction is induced in the host as a result, it may be assumed that an equilibrium between the two exists. If because of an alteration in the host, a change in the infectious agent, or some variation in both, such an equilibrium were to become upset or unbalanced, one or more of a number of possible developments might ensue. In relation to the present study, the more pertinent of these are: (1) inapparent infection of the host with the infectious agent; (2) manifest infection of the host with the infectious agent.

As has been shown in this study certain non-specific stimuli, *e.g.* the intranasal injection of suspensions of normal chick embryos, frequently induced in several species the development of specific antibodies and active immunity against a particular latent virus; *e.g.*, PVM. In terms of the considerations given above it seems reasonable to suggest that such stimuli led to this surprising result by unbalancing a preexisting equilibrium between host and latent virus; that this

was followed by an inapparent infection to which in due course the host responded; and that certain manifestations of this response were the production of demonstrable antibodies and the development of active immunity to reinfection with this agent. Thus it appears that under certain conditions, relatively innocuous procedures may, without causing any visible evidence of infection, upset an equilibrium between host and latent virus and lead to the development of a state of specific immunity.

Why some non-specific stimuli, *e.g.* native materials derived from other species, should be effective, whereas other non-specific stimuli, *e.g.* autoclaved broth, should be ineffective, is not yet known. It seems possible that the intranasal injection of undenatured material containing various antigenic substances might induce a different reaction in the host than would the similar injection of materials which had been subjected to vigorous heating. It is conceivable that this reaction might result in a sufficient, even though transient, alteration in the normal physiology of the cellular components of the respiratory tract to permit a latent virus associated with them to gain the ascendancy, and thus cause its presence to be revealed. In this connection it is of interest that Thomas and Kolb (10) have shown that the intranasal injection of fresh human serum in normal mice frequently induced the development of pneumonia and the appearance of another latent virus belonging to the psittacosis-lymphogranuloma group, whereas the similar injection of serum which had been heated at 56°C. did not. It is, of course, also possible that the latent virus itself is in some manner altered as a result of the application of appropriate non-specific stimuli, but present knowledge is insufficient to support a hypothesis in favor of this alternative.

It is evident that there is a close parallelism between the phenomenon of non-specific stimulation described in this paper, and that previously reported by Shope (11) with swine influenza. In the latter case, however, appropriate non-specific stimuli were found to have even more obvious effects, since manifest acute respiratory disease, *i.e.* swine influenza, developed in a certain percentage of pigs followings their application. It will be recalled that Shope (11) also found striking seasonal differences in the frequency with which the "provoking" stimuli he employed were effective.

In the light of the evidence presented in this paper, it is apparent that certain results obtained during 1942 and 1943 in this laboratory require reinterpretation. In the experiments previously reported (9), it was found that following the injection of specimens or chick embryo passage material derived from patients with primary atypical pneumonia in cotton rats and rabbits, antibodies against PVM developed. This fact was then considered erroneously as an indication that such specimens and passage materials contained a virus antigenically related to PVM. It is now evident that identical results can be obtained with materials known not to contain either PVM or any similar agent. Consequently the results of both the earlier and the present experi-

ments with materials from patients with primary atypical pneumonia are explicable on the basis that these specimens, like normal embryo suspensions, served merely to evoke a specific immune response to a latent virus previously harbored by the animals tested.

SUMMARY

The results of neutralization tests with PVM and serum obtained from numerous animal species indicate that antibodies against this virus were present in the blood of all mammalian species tested, as not in that of fowls, and that their incidence in various species was widely different. They indicate, also, that in certain species, particularly the cotton rat, there were marked seasonal variations in the incidence of such antibodies; in the late winter and spring the incidence was much higher than during the summer and fall seasons. Cotton rats and hamsters which did not possess neutralizing antibodies against PVM were susceptible to manifest pulmonary infection with this virus, irrespective of the effects of previous experiments upon them, whereas those which possessed such antibodies were immune. It is suggested that circulating antibodies against PVM were present as a result of preceding infection with a latent virus; either PVM or an agent closely related to it in antigenic composition.

Appropriate non-specific stimuli, *e.g.* the intranasal injection of suspensions of normal chick embryos, induced the development of neutralizing antibodies against PVM with significantly greater frequency in each of three species than occurred in control animals. Materials derived from patients with primary atypical pneumonia yielded results almost identical to those obtained with normal chick embryo suspensions. It is suggested that such materials, like the other non-specific stimuli employed, were effective in evoking a specific antibody response, because they unbalanced an equilibrium which previously existed between animal host and latent pneumotropic virus.

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