

THE RELATION OF PLEUROPNEUMONIA-LIKE ORGANISMS  
TO THE CONJUNCTIVAL CHANGES OCCURRING IN  
MICE OF THE PRINCETON STRAIN

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The association of pleuropneumonia-like organisms (PPLO) with an ocular reaction, seemingly conjunctivitis, in young mice of the Princeton strain (non-Swiss) was recently reported (1). The Princeton mice have been bred for 28 years with no additions from outside sources and have been extensively used in studies on infectious catarrh (2) and other respiratory diseases (3). The prevalence of these organisms on the conjunctiva and the nasal mucosa of young and adult mice interfered with their use in experiments of this nature. In an attempt to eradicate the infection in question a program of selection was begun in the winter of 1949. The subsequent history of the selected mice and the results of their use in studies on conjunctivitis are discussed in the present paper.

*The Development of a Selected Colony of Princeton Mice*

The selected colony was developed from 4 litters of Princeton mice which had been artificially delivered and subsequently reared by uninfected Swiss foster mothers.

The operative procedure for the delivery of the young was carried out by Dr. Carl Ten-Broeck. Immature mice were removed by cesarean section from isolated infected mothers just before the calculated date of parturition and each litter was placed with a nursing Swiss female. Examination of the Swiss colony over an extended period had indicated that these mice were free from ocular infection with PPLO (1). Losses through improper timing of the cesarean section were numerous and the rearing of the young Princeton mice proved unexpectedly difficult. Eventually, however, 4 litters were successfully raised by their Swiss foster mothers. The selected mice were maintained throughout in separate isolation quarters. The Swiss females were killed after the young were weaned and at autopsy failed to show PPLO either in conjunctival films or in cultures. Brother and sister matings were made for two generations, at least 1 litter being reared from each of the 4 groups. Examination of the respective mothers on weaning of their young showed no indication of ocular infection. The selected mice were then interbred in groups of 4 females and 1 male, and a colony limited to approximately 125 breeders was ultimately established.

Between February, 1949, when the selected colony was started, and October, 1949, when the first young mice were released for experimental purposes, 70 animals were killed and

examined. Adult mice and recently weaned young of both sexes were included in this series. Cultures from the conjunctivae of these mice and from the nasal passages of 30 were uniformly negative. Conjunctival films from 25 of the mice showed neither PPLO nor leucocytes.

In these and the subsequent experiments the conjunctival films were prepared with Wayson's stain. The cultures were made on plates of 30 per cent horse serum-nutrient agar (pH 8) containing 2500 units of commercial potassium penicillin G. The plates were sealed with scotch tape after inoculation and incubated at 37°C. for 5 to 7 days. In referring to the pleuropneumonia-like organisms the abbreviation PPLO is used and the terms conjunctival and radial type (the latter descriptive of the colony structure) are employed interchangeably.

A small group of infected breeders from the original colony of Princeton mice were held meanwhile in a separate isolation compartment. The young from these mice continued to show PPLO in films and cultures from the conjunctivae and in cultures from the nasal passages.

#### *The Appearance and Nature of Ocular Manifestations in Selected Princeton Mice*

On the basis of preceding findings it was assumed that the conjunctival carriage of PPLO had been eliminated in the selected colony. Additional observations over a period of 8 months afforded no contrary evidence. In the early development of the colony it was thought that the manifestations of ocular involvement had likewise been eliminated. As the number of mice increased it was apparent, however, that this was not the case. Young mice were observed with all the signs which had been attributed to conjunctivitis. By inspection it was impossible to distinguish between these mice and infected ones from the original colony. The only apparent difference was a quantitative one, there being fewer selected animals with obvious conjunctival manifestations. A detailed study was then made of these mice in an attempt to account for the unexpected ocular reaction.

The chief characteristic of this reaction was the accumulation of a chalky appearing fluid in one or both eyes. The fluid tended to settle in white flecks along the margin of the lids, resulting in partial closure, and in the corners of the eye, with wetting of the adjacent hair. These manifestations were most commonly observed in young mice during the early weeks after weaning and were rarely encountered in adult animals. The recently weaned mice are divided by sex into groups of 20. The incidence of the reaction varied markedly from group to group. In one series of 60 mice the rate was approximately 10 per cent. In addition to this acute reaction a considerable number of young mice showed a film of clear fluid over the eye, giving it a watery appearance quite unlike that of normal Swiss mice which were used for comparison.

Films and cultures were made from the eyes of 30 selected mice with signs of acute ocular involvement, a copious chalky fluid being obtained from all of them. Stained films showed no PPLO, relatively few normal appearing epithelial cells, few micrococci, a few leucocytes in two instances only, and marked, poorly stained, amorphous deposits. No colonies of PPLO were obtained from any of the cultures. Unstained wet mounts of the conjunctival fluid regularly showed innumerable small highly refractive globules which were stained a deep orange with Scharlach R and were undoubtedly droplets of some fatty material. They were sufficiently numerous to account for the turbidity of the fluid.

To determine whether or not the ocular reaction was communicable 3 contact experiments

were carried out with normal Swiss mice. In each experiment 5 selected mice with obvious signs of ocular involvement were placed in the same cage with 5 Swiss mice and contact was maintained for 4 to 5 weeks. The eyes of the 15 Swiss mice remained normal throughout the entire period. Five of the 15 selected mice still showed distinct manifestations on termination of the experiment. PPLO were not detectable in cultures from any of these animals at autopsy.

*The Transmission of Radial Type PPLO to Selected Mice by Direct Contact*

Having shown that the sporadic ocular manifestations of selected mice occurred in the absence of PPLO, we attempted to reestablish these organisms on the conjunctivae of young animals. The initial experiments were by direct contact, as it had been found previously that PPLO were regularly acquired by normal Swiss mice on exposure to naturally infected Princeton mice (1).

In the first series of contact experiments 3 groups of 5 young, female, selected mice, weighing 12 to 15 gm., were directly exposed to 5 young infected mice from the original colony. All of the latter mice showed signs of ocular involvement and PPLO were detectable in films. Selected mice with normal appearing eyes were used in these and all the subsequent experiments. Conjunctival cultures made from them, prior to exposure, were negative. The cages were maintained in a separate isolation room and the mice inspected at frequent intervals over a period of 4 weeks. Conjunctival films from the selected mice were examined on the 7th day and again on the 28th day. At this time cultures were made from the eyes and the first 2 groups of mice were killed. The 3rd group was held for subsequent use.

Ten of the 15 selected mice (66 per cent) showed PPLO in conjunctival films and signs of ocular involvement on the 7th day of exposure. At this time leucocytes were observed in films from 7 of the mice (46 per cent). On the 28th day of exposure PPLO were detectable in films and cultures from all of the 15 selected mice. Seven of these mice showed ocular manifestations and 6 (40 per cent) the presence of leucocytes. PPLO were also obtained in cultures from the nasal passages of the 10 selected mice of the first 2 experiments. At autopsy the lungs, middle ears, and nasal passages of these mice were normal.

A 2nd contact series was begun with experimentally infected mice and continued for 10 consecutive passages.

In each passage 5 young, female, selected mice were placed in the same cage with 5 previously exposed animals. The infected mice of the 1st passage had been in contact earlier with those of the 3rd preceding experiment. Conjunctival cultures were not made from the unexposed mice. The interval of contact was reduced to 2 weeks but since each group of 5 was used in the subsequent passage the over-all period of living together was 28 days. Otherwise the method of procedure was identical with that employed in the first series of experiments.

As indicated in Table I, 39 of the 50 selected mice (78 per cent) showed signs of ocular involvement on the 7th day of exposure. At this time extra- and intracellular PPLO were present in stained films from 44 (88 per cent) and leucocytes in 42 (84 per cent). The leucocyte count was occasionally as

high as 5 per microscopic field but was generally much lower. The films regularly showed considerable numbers of epithelial cells which were usually of the large rectangular type and abnormal in appearance, with faintly stained cytoplasm and degenerative nuclei. The smaller oval type of epithelial cell with deeply stained cytoplasm and nucleus commonly present in washings from uninfected mice was rarely observed. The large epithelial cells often contained PPLO as discrete units and colonies and also miscellaneous bacilli. The latter were unidentified but, as previously noted (1), several morphologic types were present. They varied markedly in distribution and were somewhat

TABLE I  
*Results of the Serial Passage of PPLO by Direct Contact in Selected Mice*

No. of passage*	Examination on 7th day			Examination on 28th day				
	No. of mice with			No. of mice with				
	Ocular signs	PPLO in films	Leucocytes in films	Ocular signs	PPLO in films	Leucocytes in films	PPLO in conjunctival cultures	PPLO in nasal cultures
1	4	5	2	1	5	0	5	5
2	4	4	4	4	5	4	5	4
3	4	5	5	1	5	0	5	1
4	3	5	4	2	5	2	5	4
5	3	3	3	2	5	1	5	4
6	3	4	4	4	5	3	5	5
7	4	4	5	2	5	3	5	5
8	4	4	5	1	5	2	5	5
9	5	5	5	3	5	3	5	5
10	5	5	5	2	5	5	5	5
Total . . . . .	39	44	42	22	50	23	50	43

\* 5 mice were used in each passage.

more numerous during the early passages. Their presence in the absence of PPLO was rarely noted. Micrococci, which are commonly observed on the conjunctivae of normal mice, were significantly reduced in the films from infected animals. Special methods were not employed for the detection of fat globules but it was apparent that they were commonly present.

On the 28th day of contact, when each passage was terminated, 22 of the 50 mice (44 per cent) showed conjunctival manifestations. The amount of fluid removable on aspiration was markedly reduced. PPLO were present in films from all of the 50 mice and leucocytes in 23 (46 per cent). At this time there was a noticeable decrease in the number of desquamated epithelial cells and in the number of miscellaneous bacilli. Some of the films showed a return of micrococci. Conjunctival cultures from all the exposed mice gave a heavy

growth of radial type PPLO colonies. Forty-three (86 per cent) of the nasal cultures were likewise positive but the number of colonies was much reduced, being 100 or less in 28. At autopsy the lungs, middle ears, and nasal passages were uniformly normal.

*The Dual Transmission of Conjunctival and Catarrhal Type PPLO in Selected Mice by Direct Contact*

Three strains of the catarrhal type of PPLO, associated with infectious catarrh, have been maintained in this laboratory for a period of years. The mice used for their carriage were drawn from the colony of Princeton mice before the selected group was established. In addition to the experimentally induced respiratory infection, these mice were also subject to the naturally acquired conjunctival infection. An experiment involving contact between them and uninfected selected mice was undertaken to determine whether or not the two types of PPLO were independently transmissible.

Radial type PPLO were regularly demonstrable on the conjunctivae of unselected Princeton mice which had previously been injected with the 3 catarrhal strains. Five dually infected females, one group from each strain, were placed in the same cage with 5 uninfected selected mice. Contact was maintained until chattering, indicative of the onset of infectious catarrh, was observed. With 2 of the strains the experiments were then discontinued and the exposed mice autopsied, films and cultures being made from the conjunctivae and the middle ears. With the 3rd strain contact was continued for 3 additional passages. A similar examination was made at the end of each exposure.

The results of this experiment clearly indicated that the two types of PPLO were simultaneously transmitted from dually infected mice to exposed selected ones. At the end of the respective contact periods, which varied from 62 to 102 days, all of the 30 selected mice showed organisms of the conjunctival type in ocular cultures and 24 of them organisms of the catarrhal type in middle ear cultures. Signs of ocular involvement were observed in 5, leucocytes in 6, and otitis media in 25 of the exposed mice.

*The Ocular Examination of Selected Mice Injected Intranasally with PPLO of the Catarrhal Type*

The preceding results had intimated that the two types of PPLO, which the selected mice acquired by contact, were sorted out and finally appeared only in the usual *loci*. Since the cultures from these mice generally showed innumerable colonies, it was impossible to be certain by inspection that all of them were of a given type. If ocular infection with the catarrhal type does occur under these circumstances, the organisms should be demonstrable on nasal instillation, their discharge in nasal secretions affording an opportunity for localization on the conjunctivae. To test this possibility cultures were made from the eyes and middle ears of selected mice previously injected intranasally with PPLO of the catarrhal type.

Three groups of 5 young selected mice with normal eyes were injected intranasally with each of the three catarrhal types of PPLO. The inoculum was pooled middle ear exudate suspended in 1 ml. of saline. The mice were anesthetized with ether prior to injection and approximately 0.05 ml. of the suspension was dropped on the nares with a syringe. The mice were then placed in isolation and observed at frequent intervals. After 4 to 5 weeks, chattering being observed by that time in each group, they were autopsied and examined. Films were made from the conjunctivae and cultures from both the conjunctivae and the middle ears.

The results of this experiment were uniformly negative in respect to the conjunctival localization of catarrhal type PPLO. At autopsy only 2 of the 45 injected selected mice showed signs of ocular involvement. Neither PPLO nor leucocytes were demonstrable in conjunctival films and the corresponding cultures were bacteriologically sterile. All of the 45 mice showed otitis media and granular colonies characteristic of the catarrhal type were obtained on culture in each instance.

*The Introduction of Radial Type PPLO by Nasal and Conjunctival Instillation*

As reported in the previous paper (1), attempts to establish radial type PPLO by nasal instillation and by implantation on the conjunctivae of uninfected Swiss mice were largely unsuccessful, ocular localization of the organisms being accomplished only by the multiple instillation of conjunctival exudate from infected Princeton mice. Additional experiments with the same objective were carried out on young mice from the selected colony. The methods employed and the results obtained are considered together under the following headings.

In these experiments selected mice weighing 12 to 15 gm. were used in groups of 5. The implanted washings were removed immediately before use from the conjunctivae of 3 to 5 infected Princeton mice showing PPLO in ocular films. A small volume of saline was mixed with the pooled fluids in the capillary pipette employed for aspiration. The cultures were 24 to 48-hour-old growths in 30 per cent horse serum-bouillon. They represented a pool of 4 isolations and had been transferred 3 to 12 times. The nasal injections were made in the usual way. The conjunctival implantations were all single and were performed bilaterally by expressing one drop of the exudate or culture suspension under the eyelids of mice lightly anesthetized with ether.

*Nasal Instillation.*—

Ten mice were injected with washings and 10 with cultures. At autopsy, 28 days later, the eyes, lungs, middle ears, and nasal passages were normal. Films made at this time showed neither PPLO nor leucocytes and cultures from the eyes, lungs, and nasal passages were all negative.

*Conjunctival Implantation.*—

*Washings.*—Fifteen mice were used and examined on the 7th day. Eight showed signs of ocular involvement. PPLO together with miscellaneous bacilli were present in films from all of the mice and leucocytes in 7. The conjunctival cultures were positive in each case. PPLO were again detectable in films from 5 mice reexamined on the 18th day.

*Culture.*—Fifteen mice were injected and examined only on the 7th day. At this time 3 showed some evidence of ocular involvement. Neither PPLO nor leucocytes were detectable in films and all of the 15 cultures were negative.

*Culture and PPLO-Free Washings.*—Ten mice received culture mixed with an equal volume of the conjunctival fluid from uninfected Princeton mice showing ocular manifestations. PPLO were not demonstrable in films and cultures from the pooled fluids. At examination on the 7th day, 4 of these mice showed signs of conjunctival involvement but all the films and cultures were again negative.

*Culture and Mucin.*—Ten mice were injected with culture mixed with an equal volume of mucin. A 5 per cent suspension of granular mucin (The Wilson Laboratories) was prepared in buffered saline (pH 7.4). After standing for 48 hours in the refrigerator the supernatant was removed and heated to 100°C. for 30 minutes. A pure growth of PPLO was obtained

TABLE II

*Results of the Conjunctival Implantation of Concentrated Cultures and of Subsequent Exposure in Selected Mice*

No. of experiment*	Age of culture	Transfer No. of culture	Examination on 7th day after instillation				Interval between injection and contact	Examination on 7th day of exposure			No. of mic with PPLO in culture on 14th day
			No. of mice with					No. of mice with			
			Ocular signs	PPLO in films	Leucocytes in films	PPLO in culture		Ocular signs	PPLO in films	Leucocytes in films	
	<i>hrs.</i>						<i>days</i>				
1	24	8	3	4	4	5	7	3	4	3	5
2	48	8	2	2	2	3	7	3	3	2	5
3	24	12	2	1	1	4	7	1	1	1	5
4	48	12	1	1	1	4	19	3	5	2	5

\* In each experiment 5 mice were injected and 5 additional ones exposed to them.

from the culture-mucin suspension. Four of the 10 injected mice showed conjunctival manifestations on the 7th day. Neither PPLO nor leucocytes were detectable in films and all cultures, made at this time and again on the 14th day, were negative.

*Concentrated Culture.*—In this experiment cultures of PPLO concentrated approximately 40 times were implanted on the conjunctivae of each of 5 mice in 4 groups. Twenty-four- and 48-hour-old 30 per cent horse serum-bouillon cultures of transfers 8 and 12 were spun in 10 ml. amounts for 45 minutes in an angle centrifuge at 3000 R.P.M. The sediments were resuspended in 0.25 ml. of buffered saline (pH 7.4) and used for instillation. Five normal mice were subsequently added to each of the cages, contact being established on the 7th day with 3 groups and on the 19th day with one.

The results of this experiment are summarized in Table II. On the 7th day after implantation, 8 of the 20 mice (40 per cent) showed both PPLO and leucocytes in films. Conjunctival manifestations were also detectable in 8 though with some variation in their distribution. At this time the ocular cultures from 16 of the mice (80 per cent) were positive. In 8 of these cultures, which were from mice with negative films, the number of colonies was 100 or less.

On the 7th day of contact, 13 of the 20 exposed mice (65 per cent) showed PPLO, 8 (40 per cent) showed leucocytes, and 10 (50 per cent) signs of ocular involvement. Cultures were not made until the 14th day when a heavy growth of PPLO was obtained in each instance.

The striking feature of the conjunctival films from all these mice was the scarcity of miscellaneous bacilli. Groups of micrococci were commonly observed together with innumerable PPLO, but in most instances no bacilli were detectable.

*The Isolation of Radial Type PPLO from the Conjunctivae of Wild Mice*

During the fall of 1949 and the early winter of 1950 conjunctival cultures were made from 50 house mice (*Mus musculus* L.) which were caught alive in the vicinity of the Princeton laboratories. Aside from the occasional presence of opaque areas in the lens, the eyes of these mice were normal in appearance. Pure cultures of radial type PPLO were obtained from the conjunctivae of 4 (8 per cent).

DISCUSSION

The initial transmission of radial type PPLO to young mice in an infected colony maintained by the usual methods of husbandry occurs postnatally by direct maternal contact. The rearing of one generation of young delivered by cesarean section in the absence of these organisms provided a means of interrupting the cycle of transmission and proved to be an adequate measure for elimination of the infection with PPLO. The transmission of this latter by indirect means has not been demonstrated under experimental conditions. In view of the occasional carriage of the organisms by house mice the possibility that this mode of communication exists must be borne in mind.

The manifestations of ocular involvement which originally led to the detection of the conjunctival infection were also observed in young mice from the selected colony (1). In these mice, however, there was no indication that the reaction was either inflammatory in nature or microbial in origin. The high content of fat droplets in conjunctival washings suggested rather that the manifestations were referable to the accumulation of secretions from the local sebaceous glands or possibly from the Harderian glands (4). The factors which underlie the marked increase in orbital fluid are unknown. This condition, which has been observed only in mice of the Princeton strain, might be an expression of anatomical defects in the secreting glands or more probably in the nasolacrimal ducts. It may be noted that a similar reaction was reported in 1937 in connection with studies on infectious catarrh (2).

There is no doubt that the colonization of radial type PPLO on the conjunctivae of young mice results in a slight transient irritation, the effects of which are somewhat more noticeable in Princeton than in Swiss mice. The local development of these organisms in selected animals is commonly attended by the appearance of desquamated epithelial cells and irregularly by leucocytes. This response is observed with both cultivated and naturally transmitted PPLO and may occur in the absence of secondary bacteria. The leucocytic reaction induced by organisms of the conjunctival type is insignificant how-



ever in comparison with the massive one which characterizes the growth of catarrhal type PPLO in the respiratory tract and the middle ears. Conjunctival localization of the radial type organisms in selected Princeton mice is also followed by a significant increase in the rate of ocular involvement. The cellular components of the conjunctival fluid from infected mice are generally too few in number to account for its turbidity. It seems probable that this reaction is not primarily a manifestation of conjunctivitis as was originally thought. It is believed, rather, that the reaction is largely the result of an exaggeration by PPLO of the innate tendency of young Princeton mice to accumulate sebaceous secretions in the conjunctival spaces.

#### SUMMARY

A selected colony free from ocular infection with pleuropneumonia-like organisms of the conjunctival type was developed from young Princeton mice delivered by cesarean section and reared by uninfected Swiss foster mothers. Young mice from this colony continued to show sporadic ocular manifestations similar to those observed in infected mice. This reaction was non-inflammatory in nature.

The organisms previously recovered from the altered conjunctivae of Princeton mice were established on the conjunctivae of selected mice of the special colony both by direct contact and by the local implantation of ocular washings and concentrated cultures.

The factors associated with the conjunctival reaction in infected Princeton mice are discussed.

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