AN INTRA-ORAL INOCULATION TECHNIQUE FOR THE PRODUCTION OF EXPERIMENTAL PNEUMONIA IN MICE

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(With 1 Figure in the Text)

The classical intra-nasal technique for the production of experimental pneumonia in mice is well known, but possesses certain inherent limitations. It is proposed in this paper to describe and evaluate a simple alternative.

REVIEW OF TECHNIQUES FOR THE PRODUCTION OF EXPERIMENTAL PNEUMONIA

Much effort has been devoted to the development of reliable techniques for the production of experimental pneumonia. These studies were originally designed to elucidate the genesis and evolution of pneumonia, especially the lobar variety. Since then the scope of these studies has been extended to include such diverse considerations, as the establishment of pneumotropic strains of viruses and rickettsiae, pathology of dust diseases, chemotherapeutic studies, serological problems including the protective value of immune sera, standardization of vaccines and the quantitative titration of virulence.

Gamaleia (1888), one of the pioneers in this field, produced pneumonic lesions in sheep and dogs by injecting pneumococcal cultures directly through the chest wall into the lungs. He also produced diffuse pulmonary lesions by endotracheal injections of anthrax and chicken cholera organisms. Wadsworth (1904), using similar techniques to those of Gamaleia, claimed to produce typical lobar pneumonia by intra-tracheal injections of pneumococci in rabbits previously immunized with the same strain of organism. Blake & Cecil's (1920) classical work on intra-tracheal injection of pneumococci in monkeys is well known.

Stillman & Branch (1924), using a spraying technique and Stuppy, Cannon & Falk (1929), using intra-bronchial insufflation confirmed the importance of a pre-existing level of humoral immunity in the production of the lobar type lesion. Jourdonais & Nungester (1935) and Gunn & Nungester (1936), using rats and subsequently Hamburger & Robertson (1940), using dogs, perfected the technique of direct bronchial cannulation. These authors also demonstrated the importance of mechanical factors, by showing that enhanced lesions could be produced when organisms were suspended in viscous solutions, such as mucin and starch instead of in broth or in saline. Smith, Andrewes & Laidlaw (1933), described an intra-nasal technique for the transmission of influenza viral lesions to ferrets. These authors subsequently (Andrewes, Laidlaw & Smith, 1934) were able to produce similar lesions

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in mice by intra-nasal injections of infective material under ether anaesthesia. Shortly after, Neufeld & Kuhn (1934) and Hoyle (1935) showed that lesions can be similarly produced by other viruses and a variety of pathogenic bacteria. Hoyle (1935) also showed that the nature of the lesions produced by many organisms were similar and that differences depended mainly on virulence, rather than on the nature of the organisms.

Burnet & Timmins (1937), were able to produce pneumonic lesions in mice, by intra-nasal inoculation of suspensions of *Haemophilus pertussis*. These authors pointed out that this technique affords a more reliable test of virulence than the usual methods of subcutaneous or intra-peritoneal injection, the results of which represent a summated effect of infection and toxaemia. Rudd & Burnet (1941) and van den Ende & Lush (1943) studied problems of virulence in the rickettsiae, inoculating their animals by the intra-nasal route and using a focal lesion count technique.

Increasing awareness of the dangers inherent in work of this type when human pathogens are used, has resulted in the tendency to use closed circuit systems, such as those described by Wells (1948) and Henderson (1952), in which cloud concentrations of organisms of any required density can be obtained from liquid suspensions.

Dusting techniques with erratic and unsatisfactory results were introduced by Gardner (1932), in an attempt to study experimental pneumoconiosis in guinea-pigs. Kettle & Hilton (1932) found that in order to obtain consistent lesions in the guinea-pig and rabbit it was necessary to inject directly a fluid suspension of the dust into the surgically exposed trachea.

EXPERIMENTAL

The intra-oral technique

Inhibition of the swallowing reflex in anaesthetized mice, will result in the partial inhalation of a fluid inoculum placed in the upper air and food passages.

Mice are anaesthetized with a mixture, approximately of 1 vol. ethyl chloride and 50 vol. ether. The required duration of anaesthesia is approximately 45–50 sec. The anaesthetized mouse is then held vertically by an assistant, who also retracts the head with a hooked probe, passing behind its upper incisors. The operator then opens the mouse's mouth, by traction on the lower jaw with a pair of toothed forceps (Fig. 1).

An inoculum of 0·05 ml. volume is directly deposited in the oropharynx, using a Mantoux syringe with a blunt no. 1 Luer needle, angulated to approximately 135° at about 1·5 cm. from its tip. Aspiration of the inoculum into the tracheo-bronchial tree, is attended by a characteristic moist sucking sound. The requisite plane of anaesthesia is by no means critical. Rejection of the inoculum by bubbling or spluttering is a very rare event.

The time required to inoculate one mouse is less than a minute and recovery from anaesthesia commences in a similar period of time, after which the animal can be returned to its cage.

Aerial contamination

Contamination of the atmosphere and infection of the operator can occur, either during inoculation due to spluttering, or subsequently due to the release of droplets from the already inoculated mouse. van den Ende (1943) has shown that during intra-nasal inoculation, significant droplet dissemination occurs, the extent of which depends on the smoothness of aspiration of the inoculum and the quality of the anaesthesia. van den Ende (1943) also demonstrated the considerable extent of droplet dissemination from mice after inoculation by the intra-nasal route.

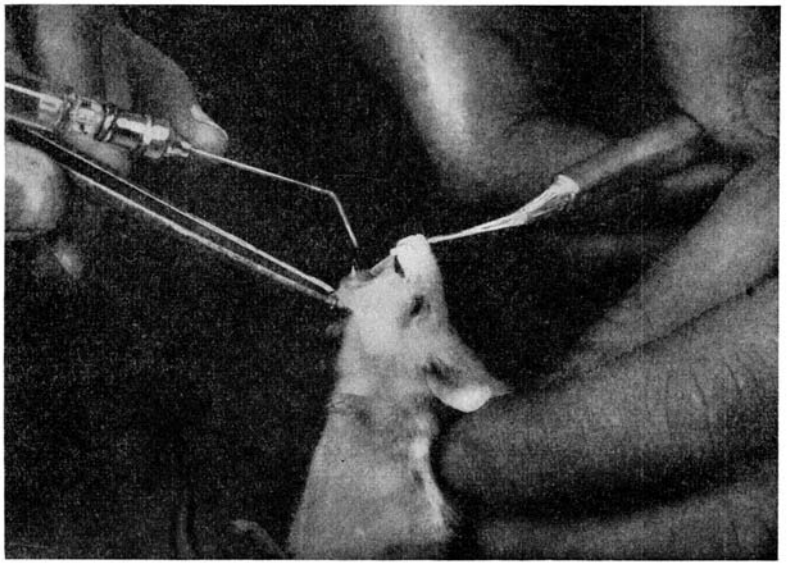

Fig. 1. Method of holding mouse prior to inoculation. The needle is advanced into the mouth up to the site of angulation before delivering the inoculum.

It was decided to compare the oral and nasal techniques, with regard to the extent of the aerial contamination occurring both during and subsequent to inoculation.

Droplet dissemination during inoculation. Droplet dissemination was assessed with a Bourdillon air sampler. The slit plate distance was adjusted to 2 mm. and the rate of air flow to 1 cu.ft./min. Gentian violet blood agar plates were rotated at a rate of 1/6 min. and were exposed for periods of 6 min. Thus, each plate received the equivalent of 6 cu.ft of air. Inoculations were performed with 0·05 ml. of a heavy suspension of Lancefield Group C Streptococci on batches of ten mice at a time, on six different occasions for both nasal and oral techniques. Protective screens were not used and the mouse receiving the inoculum was positioned 2 ft. away from the air-intake tube of the sampler, with its head on the same horizontal level. Immediately after inoculation, each mouse was placed in a separate air-tight container, to obviate the possibility of aerial contamination from inoculated animals. The duration of inoculation for each batch was spread

over the 6 min. test period. Further plates were also exposed for 6 min., before and after inoculation. The plates were counted after 48 hr. incubation. The results listed in Table 1 indicate clearly the relative safety of the two procedures.

Table 1. *Droplet dissemination during inoculation*

Expt.	Plate droplet count: Before inoculation	Plate droplet count: During inoculation	After inoculation
	(A) Oral		
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	0	0	0
6	0	0	0
	(B) Nasal		
1	0	6	1
2	0	8	2
3	0	10	2
4	0	4	1
5	0	6	2
6	0	8	3

Table 2. *Droplet dissemination after inoculation*

Time in minutes after conclusion of inoculation	Plate droplet counts: Oral	Plate droplet counts: Nasal
0–6	19	103
7–13	2	39
14–20	7	15
21–27	1	22
28–34	1	10
35–41	1	8
42–48	1	6
49–55	0	7

Droplet dissemination after inoculation. Two batches of fifteen mice were inoculated orally and nasally, respectively, with a heavy suspension of *Chromobacterium prodigiosum* on two separate occasions. Care was taken to ensure critical anaesthesia, to avoid bubbling or spluttering in the mice undergoing intra-nasal inoculation. The conditions of the air flow, slit plate distance and plate rotation, were as described previously. Nutrient agar plates were substituted for gentian violet blood agar plates.

Immediately after inoculation the mice were placed in a large cardboard box connected to the intake tube of the air sampler by a wide-bore flexible rubber tube. Air was extracted from the box and passed over a series of eight plates over a period of 55 min. Each plate was exposed for 6 min., with 1 min. interval between successive exposures. Plate counts were performed after 48 hr. incubation—24 hr. at 37° C. and 24 hr. at room temperature. Table 2 shows that with both techniques

significant droplet dissemination occurs, especially in the first 30 min. after inoculation, but the extent of this is seen to be greater with the nasal than the oral technique.

The pneumonic lesion

As Neufeld & Kuhn (1934) and Hoyle (1935) have shown, pneumonic lesions can be readily produced in the mouse by almost any mouse pathogen, including viruses, rickettsiae and protozoa besides bacteria. The histology of experimental bacterial pneumonia has been well described by Hoyle & Orr (1945). Using the technique described in this paper, pneumonic lesions can be obtained with a high degree of constancy, without even the necessity for occasional rejection of animals.

Virulence tests

In the author's hands the intra-oral method has been found to yield sufficiently consistent results, to be used for the quantitative titration of virulence. Spontaneous death of animals with demonstrable pneumonic lesions at autopsy, was taken as the critical end-point. Procedures were carefully standardized along the lines indicated by Standfast (1951).

Table 3. *4-day mortality rates* (*V.* 21)

Concentration organisms/ml.	Alive	Dead	Totals		% mortality
			Alive	Dead	
10^9	1	9	1	38	97
10^8	0	10	1	29	97
10^7	3	7	4	19	83
10^6	4	6	8	12	60
10^5	5	5	13	6	32
10^4	9	1	22	1	4
10^3	10	0	32	0	0

Calculated $LD_{50} = 5{\cdot}73 \pm 0{\cdot}32$.

Expt. V. 21. An overnight broth culture of *Klebsiella pneumoniae* Type 2. (N.C.T.C. 9503) was centrifuged. The centrifugate was washed once in saline and then resuspended in saline. Using Brown's opacity tubes a series of seven suspensions at tenfold dilutions from concentrations of 10^9–10^3 were prepared. Inoculations were effected within 1 hr. of the preparation of suspensions. No animals were rejected.

Inbred albino male mice, 4–6 weeks old, weighing 19–24 gm. were used. The animals were fed on M.R.C. diet, no. 41 and water *ad lib.* Ten animals were inoculated with each concentration of suspension.

Autopsies were performed shortly after spontaneous death occurred. Specificity of an individual death was confirmed by the presence of pneumonic lesions and positive lung cultures. Lung cultures were obtained by seeding the right middle lobe of each mouse into Robertson's meat broth.

Pneumonic lesions and positive lung cultures were obtained in all of the seventy animals inoculated. The 4-day LD_{50} was calculated according to the method of Reed & Muench (1938) and mortality rates are listed in Table 3. The calculated

LD_{50} (Probit method) was found to be $5{\cdot}73 \pm 0{\cdot}32$. Table 4 gives the corresponding data for a replicate experiment (V. 22), performed on a subsequent occasion, in which the LD_{50} was $5{\cdot}4 \pm 0{\cdot}27$. There is no statistically significant difference between these results ($P = 0{\cdot}23$).

Table 4. *4-day mortality rates* (*V.* 22)

Concentration organisms/ml.	Alive	Dead	Totals Alive	Totals Dead	% mortality
10^9	0	10	0	41	100
10^8	0	10	0	31	100
10^7	2	8	2	21	91
10^6	3	7	5	13	72
10^5	6	4	11	6	35
10^4	8	2	19	2	9
10^3	10	0	29	0	0

Calculated $LD_{50} = 5{\cdot}4 \pm 0{\cdot}27$.

DISCUSSION

van den Ende (1943), as previously mentioned, clearly demonstrated the serious danger of infection to the operator when human pathogens were used for intra-nasal inoculation of mice. He further emphasized that air-tight protective boxes must be used for inoculation under these circumstances. Boxes of this type have been used with success by van den Ende and his colleagues (see Report, 1946), in their classical studies on rickettsial diseases. Experimental evidence is presented in this paper, to show that the intra-oral technique is a simple and relatively safe procedure, provided the danger from already inoculated animals is appreciated. It is suggested that this method with elementary precautions be adopted for all except the most dangerous of human pathogens, in which case closed circuit systems are probably ideal. Apparatus of this nature is, however, costly and at the present stage its use is confined to a few specialized centres.

Unlike the intra-nasal technique, anaesthesia during intra-oral inoculation need by no means be critical to avoid spluttering or bubbling. Rejection of animals who have failed to accept their inoculum satisfactorily, is thus an extreme rarity. This, however, is by no means the case with the intra-nasal technique. Burnet & Timmins (1937) among other authors, pointed out the not infrequent necessity for rejection of animals that spluttered, or did not otherwise rapidly inhale the inoculum administered by the intra-nasal route. The facility, rapidity and smoothness with which the intra-oral inoculation can be performed, affords in the author's hands at least, a striking contrast to the difficulties of the intra-nasal technique.

Apart from the study of pneumonic lesions produced by any mouse pathogen, the intra-oral method can also be used as indicated in this paper, for quantitative virulence studies. It is also suggested that this technique may find a use in, routine virus work, experimental pneumoconiosis, standardization of vaccines such as Pertussis and possibly for the study of the action of carcinogens on the lung.

SUMMARY

Techniques for the production of experimental pneumonia are briefly reviewed. A new method of intra-oral inoculation in the anaesthetized mouse is described and its advantages with particular reference to the danger of aerial contamination and facility of technique discussed. An application of this method to virulence studies is indicated and other possible applications suggested.

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