Studies on respiratory immunization with tetanus toxoid: the role of adjuvants

By H. C. BARTLEMA, RIENTSJE BRAUNIUS AND LILY HÖLSCHER

*Medical Biological Laboratory TNO, 139 Lange Kleiweg, Rijswijk 2100,
The Netherlands*

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SUMMARY

Aerosol vaccination of mice with purified plain tetanus toxoid does not induce an immune response unless a suitable adjuvant is added.

Aluminium phosphate is without effect by aerosol treatment. Killed cells of *Klebsiella pneumoniae*, although effective, are unsatisfactory owing to the long inhalation period needed.

Killed *Bordetella pertussis* cells were found to be an excellent adjuvant. A single aerosol treatment with a toxoid-*B. pertussis* mixture during a moderate exposure period evoked a considerable immune response. With repeated aerosol treatment of primed mice the addition of adjuvant is not required; booster treatment with plain toxoid is at least as effective.

Extracts from *B. pertussis* cells exert as good an adjuvant effect as the whole-cell vaccine. The remaining cell-wall debris also appears to be an active adjuvant.

In combination with constant doses of adjuvant (10^8 *B. pertussis* cells), the 50 % protective doses (ED 50) of toxoid were determined by inhalation and by s.c. injection and were found to be 0.1875 and 0.0625 LFU respectively. This would imply that, as a result of the adjuvant action, the s.c. ED 50 is reduced by approximately a factor of 20; whereas the respiratory ED 50 is decreased by at least a factor of 100.

It is suggested that the much more pronounced adjuvant activity in aerosol immunization is associated with the induction of strong cell-mediated hypersensitivity in the respiratory tract.

INTRODUCTION

In recent years there has been increasing interest in immunization by the respiratory route. This is partly because mass vaccination could be more easily performed in this way (Fontanges, 1966) and partly in the hope that a local immunity might be established in the respiratory tract by secretory immunoglobulins (IgA type antibodies), thus producing a protective barrier against micro-organisms penetrating by this route. The idea has been supported by studies on virus vaccines (Waldman *et al.* 1968; Waldman, Mann & Small, 1969; Perkins *et al.* 1969). Most studies so far reported concern the use of killed or attenuated bacterial and viral vaccines, either in a dry or in a liquid state. Only a few investigations have dealt with toxoid. Yamashiroya, Ehrlich & Magis (1966) demon-

strated that in guinea-pigs aerosol vaccination with tetanus toxoid induced a distinct primary response which, however, was much weaker than that induced by subcutaneous (s.c.) vaccination. On the other hand, aerosol revaccination after a primary s.c. vaccination was as effective as a s.c. booster. Likewise, Wigley, Wood & Waldman (1969) found that in volunteers soluble tetanus toxoid given by aerosol as a booster treatment induced increases in serum antibody titres comparable with those resulting from a s.c. booster. An excellent booster effect of aerosol treatment with lyophilized diphtheria toxoid was observed also by Fontanges in monkeys first vaccinated by s.c. injection. Unlike the guinea-pigs studied by Yamashiroya *et al.* (1966) the monkeys also showed a very satisfactory primary response to aerosol vaccination, the calculated inhaled dose being less than that needed for a s.c. primary dose (Fontanges *et al.* 1970). Similar results were reported by Fournier in primary aerosol vaccination of monkeys with lyophilized tetanus and diphtheria toxoid (Fournier, 1969).

To obtain insight into the fundamental processes involved in respiratory immunization, studies were undertaken in our institute using tetanus toxoid as a simple antigen and mice as test animals. Syngeneic mice can be used easily in large numbers and so permit better statistical analysis of results. In addition, the cellular processes underlying the immune response to an antigen introduced by the respiratory route can, in mice, be studied at a more fundamental level since the costs involved in sacrificing large numbers of these animals are generally acceptable.

The present article describes the results of aerosol immunization with purified toxoid and the effect of aluminium phosphate (AlPO_4) and preparations of *Klebsiella pneumoniae* and *Bordetella pertussis* as adjuvants.

MATERIALS AND METHODS

Test animals

D 57 mice (F_1 hybrids from DBA males and C 57 Black females) of either sex weighing 20–25 g were used.

Vaccines and reagents

Highly purified concentrated solutions of tetanus toxoid containing 7000–10,000 Limit of Flocculation Units (LFU) per ml., lyophilized toxin, standard antitoxic serum, and *B. pertussis* preparations were obtained from the National Institute of Public Health (N.I.P.H.), Bilthoven, The Netherlands. Apart from a whole-cell vaccine with a concentration of 128×10^9 cells/ml., two other *B. pertussis* preparations were used. One was a soluble extract which was prepared in the N.I.P.H. by a procedure for manufacturing a non-toxic pertussis vaccine; the other was a suspension, mainly consisting of the cell-wall debris which remains after extraction of the bacterial cells (van Hemert, van Wezel & Cohen, 1964).

Whole-cell *B. pertussis* vaccine was mixed with plain toxoid solution to give a final concentration of approximately 7000 LFU of toxoid and 3.8×10^{10} bacterial cells per ml. of spraying liquid. The other *B. pertussis* preparations were mixed with the toxoid solutions in ratios equivalent to that of the whole-cell-toxoid mixture.

'Tetanus phosphate toxoid' (TPT) was prepared by mixing a purified toxoid solution of 3200 LFU/ml. and an AlPO_4 suspension of suitable dispersion containing 25 mg./ml. in various ratios. The mixture showing an optimum adsorption ratio was selected for use as an aerosol. It contained approximately 2000 LFU toxoid and 10 mg. AlPO_4 per ml.

An 18 hr. culture of *K. pneumoniae* in tryptose broth containing $5-6 \times 10^9$ cells/ml. was centrifuged, the cells were killed by treatment with 0.5 % formalin for 18 hr. at 4° C., repeatedly washed and resuspended to the original volume in a plain toxoid solution containing 5400 LFU/ml. The toxoid was not adsorbed to the bacterial cells as it was to AlPO_4 ; after 24 hr. agitating at 37° C. and pH 6 nearly all the toxoid could be recovered in the supernatant of the mixture.

Aerosol vaccination

Groups of 20 mice were exposed in a modified Henderson apparatus (Henderson, 1952) to aerosols sprayed with a Collison type nebulizer. By measuring the droplet size distribution of the cloud produced (May, 1945) the 'count median diameter' (CMD) and the 'mass median diameter' (MMD) were found to be 2.5 and 5 μm . respectively (Wolf *et al.* 1959). Therefore, almost all of the resulting droplet nuclei will penetrate easily into the lower respiratory tract of the test animals.

The 'absolute spray factor' (ASF), defined by Henderson as the ratio of the measured amount of substance per litre of aerosol to the amount of substance per litre of sprayed liquid, had been found for our aerosol device to have a constant value of 2×10^{-6} . Using this value and assuming a mean respiratory volume for mice of 25 ml./min. (Guyton, 1947) a rough estimate could be made of the exposure period required for any vaccine dose to be inhaled. During treatment cloud samples were taken in all-glass liquid impingers and evaluated by the indirect haemagglutination inhibition (HI) test (Stavitsky, 1954). By means of these assays the doses given were determined.

Immune assay

Immune responses were evaluated 3 weeks after immunization by s.c. challenge with 50 mouse LD₅₀ of toxin, and by antitoxin titrations in the pooled sera. All sera were titrated *in vitro* by the indirect haemagglutination (HA) technique as described by Stavitsky (1954). In addition, some sera were titrated *in vivo* by the mouse protection test on an 0.001 antitoxin unit (AU) level (J. D. van Ramshorst, personal communication).

RESULTS

Effect of plain toxoid inhalation

To find an adequate aerosol dosage range the 50 % protective dose of plain toxoid by s.c. injection (s.c. ED₅₀) was determined in preliminary experiments and found to be 1.2 LFU. Studies with ¹³¹I-labelled toxoid, not to be reported here, have shown that only 10-20 % of the estimated inhaled doses are primarily retained in the lower respiratory tract (J. L. F. Gerbrandy, to be published). Accordingly, doses in the range of 10-50 LFU were given by exposures varying from 30 min. to

Table 1. *Primary immune response of mice 3 weeks after administration of plain tetanus toxoid by aerosol or by subcutaneous injection*

Route of administration	Dose (LFU)	Survivors 5 days after challenge*	Antitoxic serum titre (haemagglutination)†
Aerosol	10-12	0/20	n.d.
	20-24	1/20	n.d.
	40-48	1/16	< 10
S.c. inj.	0.5	11/20	128
	1.0	20/20	n.d.
	2.0	20/20	320

n.d. = not determined.

* Numbers of survivors/numbers challenged.

† HA titrations of pooled sera of four animals from each group.

Table 2. *Immune response of mice 3 weeks after repeated administration of plain tetanus toxoid by various combinations of inhalation and s.c. injection*

1st dose (LFU)	2nd Dose* (LFU)	Survivors 5 days after challenge†	Antitoxic serum titre (haemagglutination)‡
10-12 (R)	10-12 (R)	1/20	n.d.
0.5 (I)	10-12 (R)	16/16	4000
0.5 (I)	0.5 (I)	16/16	4000

n.d. = not determined. (R) = given by the respiratory route. (I) = given by injection.

* Doses given 3 weeks apart.

† Numbers of survivors/numbers challenged.

‡ HA titrations of pooled sera of four animals from each group.

2 hr. Control animals were given toxoid by the s.c. route in doses of 0.5-2.0 LFU. The results of this first series of experiments are presented in Table 1.

The figures clearly demonstrate that almost no immune response is provoked in mice by inhalation of plain toxoid in doses up to 50 LFU. On the other hand, s.c. injection of as little as 0.5 LFU already induces partial protection and a measurable amount of antitoxin in the serum.

Results obtained with aerosol treatment in two doses given 3 weeks apart are given in Table 2. In the same test two groups of control mice were given a first dose of toxoid by injection. One of these groups was revaccinated by the same route, the other group was given a booster treatment by aerosol.

It can be seen that inhalation of plain toxoid on two occasions did not induce a better immune response than did a single inhalation. However, when given as a booster to mice already first vaccinated by injection, the inhaled toxoid provoked a marked anamnestic reaction similar to that induced by a booster injection.

To exclude the possibility that the poor results of plain toxoid inhalation were due to a denaturation and loss of immunogenicity by the process of aerosolization mice were vaccinated with aerosol sampling fluid by s.c. injection. No change in s.c. ED 50 as compared with the original toxoid solution was noted.

Addition of aluminium phosphate

The strong adjuvant activity of AlPO_4 in s.c. tetanus vaccination is well known. To investigate the possibility that TPT given by aerosol might also induce a good immune response 20 mice were exposed for a period of 90 min. to an aerosol of this preparation.

The presence of AlPO_4 did not interfere with the toxoid titrations by indirect HI. Determination of the ASF by toxoid titrations and by phosphate analysis (Dr Oosterbaan, Biochemical Department, Med. Biol. Lab.) yielded similar values. This indicates that phosphate and toxoid are sprayed from the mixture with equal efficiency. However, the ASF was less than for plain toxoid and diminished further during the production of aerosol. The inhaled dose of toxoid was calculated to be approximately 3 LFU. The 50 % protective dose by s.c. injection of TPT in our test mice was found to lie between 1/2 and 1/6 LFU. If the antigenic activity of the inhaled TPT had proved equal to that of the injected TPT some protective effect might have been expected. However, the results were disappointing since none of the test animals survived the challenge. An explanation for this negative result was found in a second test by measuring the droplet size distribution of TPT at the site of its emerging from the nebulizer and after it had passed through the system. The droplet size distribution in the emerging TPT aerosol did not deviate from that in plain toxoid aerosol; the CMD and the MMD being 2.9 and 4.4 μm . respectively. Pl. 1, fig. 1 is a photomicrograph showing a few TPT aerosol droplets trapped on a greased slide which was placed a few cm. from the mouth of the nebulizer. The AlPO_4 particles which are distinctly visible in these droplets were supposed to remain dispersed in the aerosol as droplet nuclei. However, microscopical examination of aerosol particles which were trapped at the end of the animal exposure tube revealed a picture as shown by Pl. 1, fig. 2. Hardly any single droplet nuclei were found. Instead, larger or smaller conglomerates of particles could be observed, the majority of which had grown to such a size that they could neither be inhaled nor penetrate very far into the bronchial tree. Conglomeration of AlPO_4 droplet nuclei following evaporation of the surrounding water may be due to electrostatic forces resulting from the charges on the micro-crystals. The mice in this second test were killed immediately after exposure. The lungs were removed *in toto* and pooled. Lungs of 20 untreated mice were collected as a control and the materials were assayed for aluminum in the 'Analytical Institute TNO' by means of activation analysis. No aluminum could be detected in the lungs of either group.

Addition of bacterial adjuvants

The addition of bacterial cells as a possible adjuvant was based on the finding in routine vaccination that when using combined bacterial and toxoid vaccine the presence of the first often increases the immune response to the latter. The particularly strong potentiating effect of *B. pertussis* on the immunogenicity of injected protein antigens has been extensively studied (Munoz, 1964). However, little is known about the effect of bacterial adjuvants in respiratory immunization.

At first, killed *K. pneumoniae*, which is a respiratory pathogen for mice, was tested. The animals were exposed to an aerosol of the toxoid-*Klebsiella* mixture for a period of 2 hr., since it was expected that the spraying efficiency of this slightly viscous fluid would be lower than that of a plain solution. From cloud sample titrations the inhalation dose was estimated to be approximately 20 LFU. Three groups of 10 mice each were vaccinated with the same mixture by s.c. injection in doses of 0.6, 1.2, and 2.4 LFU respectively.

Twelve of the 20 aerosol-treated mice (60 %) survived challenge, whereas all the injected animals survived. It is evident that *K. pneumoniae* cells had a significant effect on the immunogenicity of inhaled toxoid since most of the animals were protected. However, to afford this partial protection a long inhalation period was needed, and since the animals would hardly endure a more prolonged exposure in the Henderson apparatus a higher rate of protection might not be easily attainable.

In view of its great reputation as a bacterial adjuvant *B. pertussis* was used in subsequent tests. The ASF of the mixed toxoid-*B. pertussis* preparation was found to be the same as that for plain toxoid solution (2×10^{-6}). Three groups of mice were exposed to an aerosol for a period of 40 min. Inhalation doses were estimated to be 16 LFU of toxoid and approximately 10^8 cells. Two of the groups were given a second treatment of equal toxoid dosage 3 weeks later, one group with and one without *B. pertussis* cells. Control groups received plain toxoid treatment only. One of them was given a second treatment also. In comparative tests three groups of mice were vaccinated with the toxoid-*B. pertussis* mixture by s.c. injection of 0.5 LFU toxoid and 3×10^6 *B. pertussis* cells. Two groups had a booster injection, one with and one without adjuvant. From each group four animals were killed at the time the others were challenged, and their sera were pooled for antibody titration. Table 3 presents the results of this test series.

It is evident from the table that tetanus toxoid given by aerosol, together with *B. pertussis* cells, confers a significant degree of immunity in contrast to plain toxoid. As would be expected a booster treatment with plain toxoid provoked at least as strong a secondary immune reaction as a booster treatment with the mixture. The figures suggest also that at the given doses aerosol immunization with the toxoid-adjuvant mixture compares favourably with s.c. vaccination. The primary and secondary immune responses were at least as good as those following injection.

It has been shown by others that the adjuvant effect of *B. pertussis* extracts on the antibody response to injected protein antigens can be as potent as that of the intact bacterial cells (Farthing & Holt, 1962). In the next series of experiments the effect of two preparations of *B. pertussis* on the immunogenicity of inhaled toxoid was compared with that of the whole-cell vaccine. According to data provided by the Laboratory for Vaccine Production of the N.I.P.H. one of these preparations, the soluble extract, exhibited nearly all the biological activity contained in the original cell suspensions in terms of immunizing potency and histamine-sensitizing factor. In the other preparation almost no biological activity could be discovered.

As can be seen from the data summarized in table 4 the extract has indeed an adjuvant activity similar to that of the whole-cell vaccine. However, the cell-wall debris also appears to be an active adjuvant.

Table 3. Immune response of mice 3 weeks after aerosol or s.c. vaccination with tetanus toxoid combined with *B. pertussis* cells

Route of administration	1st dose (LFU)	2nd dose* (LFU)	Survivors 5 days† after challenge	Antitoxic serum titre‡	
				Indirect HA	Mouse test
Aerosol	16 (+ <i>B. pert.</i>)	—	15/16	128	n.d.
	16 (+ <i>B. pert.</i>)	16 (+ <i>B. pert.</i>)	16/16	16,000	1.15 AU/ml.
	16 (+ <i>B. pert.</i>)	16	16/16	n.d.	2.56 AU/ml.
Aerosol	16	—	0/16	n.d.	< 0.001 AU/ml.
	16	16	1/16	< 2	n.d.
S.c. injection	0.5 (+ <i>B. pert.</i>)	—	13/16	32	n.d.
	0.5 (+ <i>B. pert.</i>)	0.5 (+ <i>B. pert.</i>)	16/16	8000	0.80 AU/ml.
	0.5 (+ <i>B. pert.</i>)	0.5	6/6	n.d.	0.45 AU/ml.

* Doses given 3 weeks apart. † Numbers of survivors/numbers challenged.

‡ Titrations of pooled sera of four animals from each group. n.d. = not determined.

Table 4. Immune response of mice 3 weeks after single or repeated aerosol administration of mixtures of tetanus toxoid and *B. pertussis* preparations

Mixture	1st dose (LFU)	2nd dose* (LFU)	Survivors 5 days† after challenge	Antitoxic serum titre‡	
				Indirect HA	Mouse test
T + C	8	—	20/20	n.d.	n.d.
T + E	8	—	20/20	n.d.	n.d.
T + D	8	—	16/20	n.d.	n.d.
T + C	8	8	10/10	320,000	32 AU/ml
T + E	8	8	10/10	160,000	32 AU/ml
T + D	8	8	10/10	80,000	8 AU/ml

T + C: toxoid + intact cells. T + E: toxoid + extract. T + D: toxoid + cell-wall debris.

* Doses given 3 weeks apart.

† Numbers of survivors/numbers challenged.

‡ Titrations of pooled sera of 10 animals from each group; n.d. = not determined.

To make a more accurate comparison between the adjuvant effects in vaccination by aerosol and by injection, groups of mice were treated with varying doses of toxoid in combination with a constant dose (10^8 whole-cell equivalents) of *B. pertussis* extract. In the aerosol treatments the adjuvant was given immediately before the toxoid; the dosage of the latter was adjusted by varying the concentration of the spray solution and the period of inhalation. For s.c. injections the required doses of toxoid and adjuvant were mixed and injected in 0.5 ml volumes.

From the challenge survivals given in Table 5 the 50 % protective dose for both the s.c. and respiratory route was calculated according to Litchfield & Wilcoxon (1949). These values were for the aerosol route 0.1875 with 95 % confidence limits of 0.26 and 0.14 and for the s.c. route 0.0625 with 95 % confidence limits of 0.1 and 0.04. Figure 1 shows the log. dose-response lines, both of which have a b-(slope)-value of 4.5.

The s.c. ED₅₀ of plain toxoid was determined in previous studies at 1.2 LFU.

Table 5. *Survival rates of mice challenged 3 weeks after immunization with varying doses of tetanus toxoid in combination with a constant dose of B. pertussis extract, by aerosol or s.c. injection*

Aerosol		s.c. injection	
Toxoid dose (LFU)	Survivors* 5 days after challenge	Toxoid dose (LFU)	Survivors* 5 days after challenge
0.125	2/10	0.031	1/10
0.25	7/10	0.062	6/10
0.5	10/10	0.125	9/10
1.0	10/10	0.25	10/10

* Numbers of survivors/numbers challenged.

Text-fig. 1. Log. dose-response relationships in s.c. and aerosol vaccination with tetanus toxoid combined with *B. pertussis*.

It appears that this value can be lowered by a factor of the order of 20, if sufficient adjuvant is added. The respiratory ED₅₀ of plain toxoid could not be evaluated by aerosol treatment but, according to the data already presented, it is likely to be considerably higher than 20 LFU. It would follow that the addition of adjuvant here results in a decrease of the ED₅₀ value by at least a factor of 100. The indication given already by the preceding experiments (Table 3) that the adjuvant effect is much stronger in immunization by inhalation than by injection is thus fully confirmed.

DISCUSSION

The present study clearly shows that in our experimental model single or repeated inhalation of purified tetanus toxoid in doses up to 50 LFU does not provoke an appreciable immune response in mice. An explanation for these negative results was found in separate studies which demonstrated that plain toxoid given to mice by the respiratory route induces partial unresponsiveness to subsequent injection of the same antigen (Bartlema & Braunius, 1969). It is suggested that this development of tolerance is associated with a gradual and prolonged resorption of the soluble antigen which is distributed over the large surface area of the pulmonary epithelium. The local concentration of the resorbed toxoid would then be too low for it to be effectively phagocytosed and concentrated by macrophages, thus enhancing a tolerogenic effect (Mitchell & Nossal, 1966; Cohn, 1969). It is also conceivable that the inhaled toxoid is subject to a process of 'biological filtration' (Frei, Benacerraf & Thorbecke, 1965) by lung macrophages, resulting in the removal of that part of the antigen which is responsible for its 'intrinsic adjuvanticity' (Dresser, 1968) and, consequently, in a shift of the balance between immunogenic and tolerogenic activity in the resorbed moiety.

To explain the discrepancy between our results and those of others two factors should be considered: (1) the type of test animal, and (2) the purity of the antigen.

The first factor may account for Yamashiroya's observation of a distinct, although weak, primary immune response in guinea-pigs after aerosol treatment with purified tetanus toxoid (Yamashiroya *et al.* 1966). On the other hand, Leclercq (1971), in a recent study on aerosol vaccination of mice with purified toxoid, found results which were very similar to ours in that no antibody production was seen unless a suitable adjuvant was added.

Both factors may be involved in the excellent results of respiratory immunization of monkeys with crude toxoid preparations reported by French authors (Fournier, 1969; Fontanges *et al.* 1970). Here the impurity of the vaccine probably constituted a high degree of 'extrinsic adjuvanticity' (Dresser, 1968).

In our experiments the addition of an adequate adjuvant to the inhaled toxoid changed the type of immune response from tolerance to antibody formation. This has been described for protein antigens like bovine serum albumin – a 'weak immunogen' – when given by intraperitoneal injection in a well-defined dosage scheme (Mitchison, 1964). For toxoid the phenomenon may be strictly associated with aerosol administration.

The failure of AlPO_4 to bring about this switch in immune response may be due entirely to the method of administration, i.e. by aerosol. Of the bacterial adjuvants tested the *B. pertussis* preparations had a remarkable effect which could be roughly estimated in terms of reducing the respiratory ED50 of plain toxoid by at least a factor of 100. This approximate evaluation was confirmed in later experiments where ED50 values were more precisely determined by intratracheal instillation (J. L. F. Gerbrandy, to be published).

Studies reported by others have yielded evidence that bacterial adjuvants such as Freund's complete adjuvant or *B. pertussis* exert their activity by stimulation

of thymus-derived lymphocytes which would imply that both antibody production and cell-mediated hypersensitivity to the thymus-dependent protein antigen are promoted (Allison & Davies, 1971). This might provide an explanation for the strong adjuvant effect of *B. pertussis* by aerosol treatment. M. P. C. Karelse (to be published) has observed the appearance of excessive cellular infiltrations in the lung after *B. pertussis* administration. These would favour an effective recruitment and stimulation of T-lymphocytes which in turn would promote a state of cell-mediated hypersensitivity but also, through cooperation with B-lymphocytes, would strongly potentiate the antibody response (Roitt *et al.* 1969).

That respiratory vaccination with toxoid combined with *B. pertussis* may provoke a high degree of cell-mediated hypersensitivity to the protein antigen was strongly suggested by our finding that mice, vaccinated in this way and boosted with a small dose of toxoid only, displayed a marked cellular immunity, manifested by their increased resistance against *L. monocytogenes* infection (cf. Dodd, 1970). Ten mice were vaccinated (by intratracheal instillation) with 2 LFU of toxoid and 6×10^8 *B. pertussis* cells. After 3 weeks a second dose of 1 LFU was given by the same route and 2 days later the animals were challenged by i.p. injection of 9×10^5 cells (ca. 25 LD₅₀) of virulent *L. monocytogenes*, along with a control group that was vaccinated with comparable doses by s.c. injection. Nine of the intratracheally vaccinated mice survived, whereas all animals in the control group died. This observation of an enhanced cell-mediated reactivity also would imply that primary respiratory immunization with a thymus-dependent protein antigen and a bacterial adjuvant would not be advisable in view of an increased risk of untoward side effects.

Other factors may be involved as well in the remarkable adjuvant activity displayed by *B. pertussis* in our experimental model. The adjuvant may stimulate the phagocytic or antigen-processing potency of lung macrophages or may enhance the transport of antigen to the draining lymph nodes. A more conclusive explanation of the phenomena observed in the intact animals requires insight into the cellular processes involved in the reactions to the introduction of antigens and adjuvants by the respiratory route. Histological and immunological studies both *in vivo* and *in vitro* were undertaken to explore these processes and will be reported in subsequent articles.

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EXPLANATION OF PLATE

Fig. 1. AlPO_4 -toxoid aerosol droplets emerging from the nebulizer. AlPO_4 particles are visible within the droplets.

Fig. 2. Conglomerates of droplet nuclei formed during passage through the Henderson apparatus.

Fig. 1

Fig. 2