ATTEMPTS TO POTENTIATE IMMUNITY TO INFLUENZA IN MICE

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Fazekas de St Groth and co-workers in a series of studies (1950, 1951) adduced evidence that the protective effect of influenza virus vaccines in mice could be enhanced by a simultaneous intranasal inoculation of an adjuvant. This adjuvant could be another influenza virus (for example, B virus if the vaccine was a formolized A virus) or a substance like potassium metaperiodate. They found that the adjuvant did not affect the titre of the circulating antibody; but the titre of antibody in the bronchial washings was higher in mice which had received vaccine plus adjuvant. They postulated, therefore, that the enhancement of immunity by a non-specific adjuvant was due to the increase in specific anti-haemagglutinin content of the bronchial washings. This phenomenon they called pathotopic potentiation and they believed that the adjuvant increased the permeability of the barrier between the circulation and the lumen of the respiratory tract (Fazekas de St Groth, 1951). As a preliminary to further study of this phenomenon we tried to reproduce these protection experiments without carrying out any titrations of antibody. We have been unable to demonstrate any potentiation of immunity, however, using either heterologous virus or potassium metaperiodate as a potentiating agent.

MATERIALS AND METHODS

Viruses. Influenza A, Melbourne strain. Influenza B, Lee and Crawley (England 1946) strains.

Preparation of virus. Ten-day-old fertile eggs were inoculated intra-allantoically with the appropriate virus; the allantoic fluids were harvested after a further 2 days' incubation at 35° C. Fluids were used within 24 hr. Vaccines were prepared by addition of formaldehyde (to give a final concentration of 0·05%) to fresh allantoic fluid followed by incubation at 37° C. for 24 hr. In this way the haemagglutinin titre was not modified significantly, but the infective power was abolished.

Haemagglutinin titration (HA). Serial twofold dilutions of the virus suspension were made in normal saline in 0·25 ml. volumes and an equal volume of 0·5% fowl red cells suspension was added to each dilution. The cells were allowed to settle at room temperature and readings were made of the sedimentation patterns. The titres were expressed as the reciprocal of the initial dilution of virus at the partial agglutination end-point.

Potassium metaperiodate was used M/100 in normal saline.

P strain of white mice† was used when the mice were 6 weeks old and of 12–14 g. weight.

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† A strain of mice maintained at the National Institute for Medical Research.

As far as possible the techniques used were identical with those described by Fazekas de St Groth & Donnelley (1950*a*, *b*). Briefly, the technique of an experiment was as follows: batches of five 6-week-old mice were immunized by intraperitoneal inoculation of formolized influenza virus. Some mice received at the same time an intranasal inoculation (0·05 ml.) under light ether anaesthesia of the pathotopic adjuvant under test. Eleven days later these mice and a control group of untreated mice were challenged with tenfold dilutions of active virus and killed 7 days later in order to estimate the lung lesions. The lesions were measured on an arbitrary scale the maximum response being given a value of 5·0.

The results were analysed by two different methods:

Method 1. The protection '*P* value' was calculated from the formula described by Fazekas de St Groth & Donnelley (1950*a*).

Method 2. The results of each group of mice were analysed by standard probit methods and the ED_{50} (effective dose, i.e. the log dose of challenging virus producing 50 % lung consolidation) calculated. One recent description of this method of calculation is that of Burn, Finney & Goodwin (1950).

RESULTS

The results of one experiment designed to demonstrate pathotopic potentiation are shown in Table 1.

Table 1. *Results of a single experiment showing the average lung lesions for each set of five mice*

(The lesions were measured on an arbitrary scale the maximum response being given a value of 5·0.)

Dilution of challenging virus	Average lung lesions			
	Group 1, vaccine alone	Group 2, vaccine plus nasal adjuvant	Group 3, nasal adjuvant alone	Group 4, control
10^{0}	4·73	3·22	4·83	4·90
10^{-1}	1·13	1·59	4·80	4·90
10^{-2}	0·76	0·26	4·00	4·40
10^{-3}	0·00	0·00	3·91	4·33
10^{-4}	0·00	0·00	1·92	2·44
10^{-5}	0·00	0·00	2·08	2·26
10^{-6}	—	—	1·18	0·73
10^{-7}	—	—	—	0·33
	P value $= 2{\cdot}7 \times 10^{6}$ $ED_{50} = -0{\cdot}836$ $\pm 0{\cdot}298$	P value $= 8{\cdot}1 \times 10^{6}$ $ED_{50} = -0{\cdot}430$ $\pm 0{\cdot}413$	P value $= 2{\cdot}9$ $ED_{50} = -4{\cdot}249$ $\pm 0{\cdot}647$	P value $= 1$ $ED_{50} = -4{\cdot}361$ $\pm 0{\cdot}491$

Vaccine = 200 A.D. of formolized A/Melbourne strain given intraperitoneally. Nasal adjuvant = 100 A.D. of formolized B/Lee strain. Control groups received nothing before challenge. Challenging virus = active A/Melbourne strain. A.D. = agglutinating dose.

Further experiments were carried out using B/Lee or A/Melbourne viruses alternating as vaccine or adjuvant. In other experiments the mice were vaccinated with formolized Melbourne virus, the adjuvant being potassium metaperiodate

which, according to Fazekas de St Groth, was the most effective pathotopic adjuvant. The ED_{50} for each group of each experiment, together with its standard error, is shown in Table 2. Within a given experiment, no significant difference was detected between the ED_{50} for groups of mice receiving vaccine alone and those where adjuvant also was given.

Table 2. *ED_{50} and its standard error for each group*

Expt.	Intraperitoneal vaccine	Intranasal adjuvant	Challenging virus	ED_{50} Group 1, vaccine alone	Group 2, vaccine + adjuvant	Group 3, adjuvant alone	Group 4, control
I	Lee 250 A.D.	F/Melbourne 250 A.D.	Lee	−0·337 ± 0·867	−0·596 ± 0·569	−4·765 ± 0·371	−4·378 ± 0·392
II	F/Melbourne 2000 A.D.	F/Crawley 250 A.D.	Melbourne	>0	>0	−2·999 ± 0·759	−4·341 ± 0·893
III	F/Lee 250 A.D.	F/Melbourne 500 A.D.	Lee	>0	>0	−3·562 ± 0·745	−3·143 ± 0·580
IV	F/Melbourne 300 A.D.	F/Lee 130 A.D.	Melbourne	−0·01 ± 0·638	>0	−5·25 ± 0·868	−4·95 ± 0·356
V	F/Lee 700 A.D.	F/Melbourne 600 A.D.	Lee	>0	>0	−1·97 ± 0·822	−3·35 ± 0·515
VI	F/Melbourne 200 A.D.	KIO_4 M/100 0·05 ml.	Melbourne	>0	−2·29 ± 0·574	−7·27 ± 3·08	−5·20 ± 0·425
VII A	F/Melbourne 200 A.D.	KIO_4 M/100 0·5 ml.	Melbourne	−0·608 ± 0·730	−0·998 ± 0·329	−5·61 ± 1·28	−5·41* ± 0·408
VII B	F/Melbourne 200 A.D.	KIO_4 M/100 0·05 ml.	Melbourne	−0·489 ± 0·638	−0·916 ± 0·414	−6·17* ± 1·88	−5·67 ± 0·443
VII A+B	F/Melbourne 200 A.D.	KIO_4 M/100 0·05 ml.	Melbourne	−0·556 ± 0·465	−0·972 ± 0·267	−6·47 ± 1·61	−5·52 ± 0·400
VIII	F/Melbourne 200 A.D.	F/Lee 100 A.D.	Melbourne	−0·836 ± 0·298	−0·430 ± 0·413	−4·249 ± 0·647	−4·361 ± 0·491

* These values have been calculated omitting the responses to the two highest doses as these gave a maximum effect. F/=formolized.

In three experiments (II, III and V) no conclusion can be drawn. Indeed, the protection obtained in the two groups of mice which received either vaccine alone or vaccine plus adjuvant was so great that it was impossible to estimate the ED_{50}. In these experiments we used an amount of vaccine of the same order as that used by Fazekas de St Groth and his co-workers, but Fazekas de St Groth has pointed out (personal communication) that our 6-week-old mice weighed only 12–14 g. while mice of the same age which he used weighed 22–23 g., and this may account for the high protection achieved by vaccine alone in the experiments recorded here.

Table 3 shows the protection (P value) obtained in these same experiments and calculated by method 1. Like the previous procedure, this does not show any enhancement of immunity by pathotopic vaccination.

Table 4 shows in detail the calculation of protection (P). The data given in Table 1 have been used for this calculation. First the average lesion for each group of five mice is calculated, e.g. the individual responses to 10° dilution of challenging

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Table 3. *Protection values (P) for each group*

Expt.	Group 1	Group 2	Group 3	Group 4
I	$6{\cdot}3 \times 10^5$	$2{\cdot}1 \times 10^5$	<1	1
II	$3{\cdot}6 \times 10^9$	$1{\cdot}5 \times 10^{13}$	$6{\cdot}5 \times 10^2$	1
III	$4{\cdot}8 \times 10^3$	$9{\cdot}7 \times 10^3$	<1	1
IV	$3{\cdot}1 \times 10^4$	$3{\cdot}0 \times 10^4$	1·7	1
V	$9{\cdot}7 \times 10^5$	$1{\cdot}9 \times 10^5$	6·8	1
VI	$1{\cdot}7 \times 10^4$	$1{\cdot}0 \times 10^2$	2·2	1
VII A	$1{\cdot}8 \times 10^8$	$3{\cdot}2 \times 10^8$	1·1	1
VII B	$9{\cdot}8 \times 10^5$	$4{\cdot}7 \times 10^5$	9·3	1
VII A + B	$1{\cdot}4 \times 10^8$	$1{\cdot}0 \times 10^8$	3·9	1
VIII	$2{\cdot}7 \times 10^6$	$8{\cdot}1 \times 10^6$	2·9	1

virus in group 1 were 5, 5, 5, 5 and 3·66 with an average of 4·733. These average lesions for each dilution of virus are shown in Table 1. Each average is then expressed as a percentage of 5 (the maximum response) and the corresponding probits (denoted as L_x) entered in Table 4. Taking the same example, 4·733 is 94·67 % of 5 and the probit of 94·67 % is 6·62.

The probits are summed for each group (ΣL_x) and the differences between each group and the corresponding set of controls (d_x) calculated. In the control group responses to two further dilutions are obtained in order to estimate d_{10}, i.e. the difference in ΣL_x which would be equivalent to a tenfold reduction in infectivity.

Protection is defined as antilog (d_x/d_{10}).

Table 4. *Calculation of protection values (P) for experimental results shown in Table* 1

	Dilution of challenging virus	Group 1 10^0–10^{-5}	Group 2 10^0–10^{-5}	Group 3 10^0–10^{-5}	Group 4 (controls) 10^0–10^{-5}	10^{-1}–10^{-6}	10^{-2}–10^{-}
Probit of average lung lesions $= L_x$	10^0	6·62	5·37	6·84	7·05	—	—
	10^{-1}	4·25	4·53	6·75	7·05	7·05	—
	10^{-2}	3·98	3·38	5·84	6·18	6·18	6·18
	10^{-3}	0·00	0·00	5·78	6·11	6·11	6·11
	10^{-4}	0·00	0·00	4·71	4·97	4·97	4·97
	10^{-5}	0·00	0·00	4·79	4·88	4·88	4·88
	10^{-6}	—	—	—	—	3·95	3·95
	10^{-7}	—	—	—	—	—	3·50
Sum of probits $= \Sigma L_x$	—	14·85	13·28	34·71	36·24	33·14	29·59
Difference from control $= 36{\cdot}24 - \Sigma L_x = d_x$	—	21·39	22·96	1·53	0·00	3·10	6·65 $d_{10} = 3{\cdot}32$
Reduction in lesions $= dx/d_{10} = \log P$	—	6·433	6·905	0·460	0·000	0·932	2·000
Protection $= P$	—	$2{\cdot}7 \times 10^6$	$8{\cdot}1 \times 10^6$	2·9	1·0	8·6	100·0

A difference is observed between the P values for series A and B of the seventh experiment in both groups 1 and 2 (groups $7A_1$ and $7A_2$ $P = 10^8$; groups $7B_1$ and $7B_2$ $P = 10^5$). However, the mice in these two series were inoculated at the same time and with the same material and the corresponding ED_{50}'s were similar. The ΣL_x for series A and B were also similar (ΣL_x for $A_1 = 18{\cdot}35$, for $A_2 = 17{\cdot}74$, for $B_1 = 17{\cdot}30$, for $B_2 = 18{\cdot}71$).

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The discrepancy between the P values can be explained by the fact that in method 1 the d_{10} (which is virtually the slope of the log dose-probit line) of the controls is used in the calculation of P for each of the other groups in the series.

The control group for series B gave responses which could be well fitted on a log dose-probit line. The d_{10} for this group was 4·4. The responses for the control group of series A fitted a line of very similar slope apart from one point, the response to the dose of 10^{-1}, which no doubt was due to some chance mishap and fell short of the general trend of the other points (10^{0} and 10^{-2} both gave a maximum response). A d_{10} of only 2·3 was calculated for this series, a reduction of a half due to one misfitting point.

Great care, therefore, should be taken when applying method 1 to see that there is linearity for each log dose-probit line and also that the slope of the control line does not differ appreciably from the slopes of the other groups within each experiment.

When method 2 is used this difficulty does not arise, as the ED_{50} for any particular group is found by using the fitted slope of *that* group, after it has been established that there are no significant departures from linearity.

The control group for series A did in fact give a significant departure from linearity, and it was thought justifiable to repeat the calculation omitting the two highest doses. The other groups in the series were of course unaffected.

Fazekas de St Groth (1951) showed that materials which he had found to act as pathotopic adjuvants of immunity when inoculated intranasally into mice, also had the property of enhancing the lung lesions of mice given an intraperitoneal inoculation of live influenza virus. Conversely, materials which were ineffective as pathotopic adjuvants showed no effect when given by this second technique. Fazekas de St Groth postulated that the adjuvant acted as pathotopic adjuvant by increasing the permeability of the barrier between the circulation and the lumen of the respiratory tract in the same way that he had demonstrated by this second technique an increased permeability to influenza virus.

In view of our inability to demonstrate pathotopic potentiation it seemed worth while to see whether an increased permeability to virus could be demonstrated: 1600 agglutinating doses of active Melbourne virus (greater than 10^{6} LD_{50}) were injected intraperitoneally and groups of these mice were inoculated intranasally with potassium metaperiodate, saline or nothing. No lung lesions occurred in any of the groups.

DISCUSSION

Eight experiments, including one double experiment, were performed to ascertain the enhancement of immunity by pathotopic adjuvants.

As far as possible we have operated under the same experimental conditions as Fazekas de St Groth. The same techniques of vaccination of mice and of estimation of lung lesions were employed. Virus differences cannot account for the discrepant results since we have used the same strain of Melbourne virus as Fazekas de St Groth and potassium metaperiodate as adjuvant.

The standard errors of the ED_{50} obtained in calculation of Expts. I, IV, VI, VII and VIII usually ranged from $\pm 0{\cdot}2$ to $\pm 0{\cdot}6$. Though some greater errors were

found, all but two appeared in the groups of mice receiving the adjuvant alone without vaccine (groups 3 from Expts. IV, VI, VII A, B and A+B) where the 50% lesion doses were determined by extrapolation because the lowest dilution used (10^{-5}) produced more than 50% lung consolidation. In the only titration of infectivity published by Fazekas de St Groth & Donnelley (1950*a*) which gives the individual experimental results the standard errors of the ED_{50} were respectively $\pm 1{\cdot}049$ within the group of mice vaccinated intraperitoneally and $\pm 0{\cdot}587$ in the control group. The variability of our results does not, therefore, seem to be greater than that of Fazekas de St Groth. The most likely difference between our results and those of this author appears to be in the mice used. He used 6-week-old 22–23 g. mice while ours (P strain) weighed only 12–14 g. at 6 weeks. This increase in the ratio between the amount of virus injected and the body weight may perhaps explain the fact that with doses of vaccine similar to or greater than those we employed in Expts. I, IV, VI, VII and VIII, Fazekas de St Groth and his co-workers found smaller differences between the ΣL_x in the controls and vaccinated groups than we did.

As a result of differences in weight or breed, the mice we have used would seem to differ from those used by Fazekas de St Groth and his co-workers in two respects. First, our mice showed no evidence that a non-specific adjuvant could increase their immunity to influenza as a result of vaccination. Secondly, our mice did not develop lung lesions after intraperitoneal inoculation of large amounts of active influenza virus together with intranasal inoculation of adjuvant. These findings lead us to conclude that the phenomena of pathotopic potentiation and increased permeability of the circulatory lung barrier to antibody and influenza virus are not of universal application.

SUMMARY

Under the conditions of our experiments, the local potentiation of immunity to influenza reported by Fazekas de St Groth and Donnelley could not be demonstrated.

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