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The Immune Response to Influenza Virus

I. EFFECT OF THE ROUTE AND SCHEDULE OF VACCINATION ON THE TIME COURSE OF THE IMMUNE RESPONSE, AS MEASURED BY THREE SEROLOGICAL METHODS

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Summary. The influence of the route and schedule of vaccination on the time course of the immune response was studied in groups of rabbits injected with influenza virus antigens. The route and schedule of inoculation determined the maximal level of serum antibody. The routes can be ordered intravenous, intraperitoneal and subcutaneous in descending order of efficiency. Multiple doses improved the levels of antibody by all routes, while antigen in Freund's incomplete adjuvant suppressed the initial response. Changes in the ratios between the three tests indicated differences in avidity of antibody produced during the initial and the secondary responses.

INTRODUCTION

The existence of two major theories to explain the same phenomenon can only mean that there is a fundamental lack of information to explain the phenomenon. Such is the state of affairs with antibody production; a single body of information is interpreted from fundamentally different points of view and one is presented with two theories that account for most of the facts but happen to be mutually exclusive. When such a state of affairs as this exists the immediate task in immunological inquiry is not so much the design and performance of crucial tests aimed at eliminating one or the other contending hypothesis but the systematic collection of data, comprehensive enough to cover the implications of present hypotheses and coherent enough to guard against ambiguities in the future.

Influenza virus was chosen as a natural antigen on which to establish such a reference system. This antigen is readily available, occurs in a large number of variant forms, and perhaps most important of all can be tested for with ease by many serological and immunochemical techniques. The elementary particle is characterized by a repeating antigenic area (Fazekas de St. Groth and Webster, 1963), and it can be detected by a variety of *in vitro* and *in vivo* methods. A point was made of using several of these methods side by side, since some of them measure predominantly the avidity of antibodies and others the quantity. Some of the methods are more sensitive to inhomogeneity in the reagents, others less so; some are influenced by the rate of antigen–antibody combination, whereas others depend on the equilibrium between free and combined sites.

The first paper in this series deals with the results obtained from three basic serological techniques for describing the time course of the immune response; and the dependence of

this response on the route and schedule of vaccination, as well as on the nature of the antigen.

MATERIALS AND METHODS

Diluents

Calcium–magnesium–saline, prepared according to Fazekas de St. Groth, Graham and Jack (1958), was used as buffered saline throughout. The standard medium (SM) used in infectivity and neutralization tests was prepared according to Fazekas de St. Groth and White (1958).

Virus strains

The three strains of influenza virus used were MEL (Burnet, 1935), a typical A strain; LEE (Francis, 1940), the prototype of B strains; and SW (strain 15 of Shope, 1931), the classical strain of type A swine influenza. MEL and SW show some cross reaction.

Growth and concentration of virus

About 10^3ID_{50} of virus was inoculated into the allantoic cavity of 11-day-old chick embryos. After 2 days' incubation at 35° in the case of MEL and SW, and 3 days in the case of LEE, the allantoic fluid was harvested and concentrated ten-fold by one cycle of adsorption and elution from 2 per cent human red cells. The concentrated virus suspensions were preserved with 0·08 per cent sodium azide and stored at 4°.

Titration of virus

Haemagglutinin titrations were carried out as described by Fazekas de St. Groth and Graham (1954) but 5 per cent chicken cells were used instead of 10 per cent and the tests were carried out in plastic trays. Viral infectivity was assayed in surviving pieces of allantois-on-shell, according to the method of Fazekas de St. Groth and White (1958).

Preparation of non-infective vaccines

Three methods were used to abolish the infectivity of virus preparations.

Heat treatment consisted of incubation of the virus concentrates in a water bath at 50° for 1 hour.

Ultraviolet (UV) treatment was preceded by dialysis of the virus preparations against 1000 times their volume of buffered saline for 24 hours, to remove urates and other UV-absorbing material. Then 10 ml volumes were exposed in 9 cm diameter Petri dishes for 5 minutes to the radiation from a 15 watt Phillips ultraviolet tube, at a distance of 20 cm. To ensure uniform exposure during irradiation, the preparations were mixed constantly with a magnetic stirrer.

For ultraviolet and formaldehyde treatment, the UV-treated preparations were made up to a final concentration of 0·03 g/100 ml in 40 per cent formaldehyde in water and kept at 20° for 48 hours. Residual formaldehyde was removed by dialysis against several changes of buffered saline.

The vaccines inactivated by the three methods described above were tested for residual infective virus, using pieces of surviving allantois-on-shell. As the inactivated vaccines contained high titre haemagglutinin it was necessary to transfer each piece of allantois after an adsorption period of 3 hours, in order to avoid haemagglutination by unabsorbed virus. The pieces of allantois-on-shell were removed individually with fine forceps, washed in 2·0 ml volumes of standard medium and transferred to a second tray containing standard medium and a freshly cut piece of allantois-on-shell. In other respects, the

period of incubation and the scoring of infectivity were the same as in orthodox virus assays. No infective virus could be detected by this method in any of the vaccines.

Preparation of adjuvant vaccines

Concentrated virus was made up in SM to contain 10,000 haemagglutinating (HA) doses per 0·25 ml, i.e. at double strength compared to the standard concentration of 5000 HA doses per 0·25 ml in the non-adjuvant vaccines. (One HA dose is the dose of virus giving partial agglutination (15 per cent dimers) with 10^7 chicken red blood cells.) An equal volume of Freund's incomplete adjuvant (Difco) was added to these preparations, and the mixture emulsified in a VirTis homogenizer running at maximum speed for 5 minutes at 0°. The resulting emulsion was stable: it did not spread or separate when an aliquot was dropped on to the surface of water. The water-in-oil emulsions were injected within a few hours of preparation, in 0·25 ml doses. The dose of antigen administered was thus the same as that given (in the form of aqueous suspensions) to animals in other groups.

Experimental animals

Adult outbred rabbits of several breeds (Australian wild, Belgian giant, and New Zealand white) and of both sexes were housed in individual cages with unrestricted access to a pelleted complete diet and water. Additional green fodder was given three times a week.

Three rabbits made up each treatment group. Each of the animals was bled 2 days before vaccination and at intervals thereafter. About 15 ml of whole blood was collected from the ear vein, allowed to clot at room temperature and then incubated for 1 hour at 35°. The separated sera were spun free of red blood cells, distributed in small screw-capped containers and stored at −15° without preservative.

Titration of antibody

Neutralization tests were carried out according to Fazekas de St. Groth, Withell and Lafferty (1958). Non-inactivated sera were diluted in 3·16-fold steps in 1·08 ml volumes of SM and cooled to 0° before adding 0·05 ml of SM containing the appropriate dose of virus (between 10^3 and $10^4 ID_{50}$). The actual concentration of virus varied from one strain to another, but was constant for any one strain. The virus–serum mixtures were pre-incubated for 30 minutes at 0° and then 0·05 ml of each mixture was added to each of eight replicate cups containing a square of allantois-on-shell in 0·35 ml of SM. Control titrations of viral infectivity were carried out at the beginning and end of each experiment. The trays were incubated for 60–64 hours at 35° with constant shaking. The endpoint of neutralization was judged by agglutination of one drop of 5 per cent chicken red cells at the end of the incubation period. Neutralizing potencies (pN) were calculated according to Fazekas de St. Groth (1961).

Antihaemagglutinin tests were performed in plastic trays using 0·25 ml of saline as dilution volume, four haemagglutinating doses of the homologous virus as antigen and 0·025 ml of 5 per cent chicken red cells as indicator (Fazekas de St. Groth *et al.*, 1958). The red cells used in the antihaemagglutinin tests were taken from a single donor and were insensitive to non-specific inhibition when tested with receptor destroying enzyme (RDE) treated normal rabbit serum. All sera both normal and immune were treated with RDE before use to destroy nonspecific inhibitors of haemagglutination (Fazekas de St. Groth, 1949).

Complement fixation tests followed the overnight procedure of Fazekas de St. Groth *et al.* (1958). Sera inactivated at 62·5° for 20 minutes were diluted in 0·25 ml volumes of Ca–Mg–saline. Then a standard drop (0·025 ml) containing 8 units of antigen and 3 HD_{50} of complement was added to each cup, and the trays were incubated at 4° for 16–20 hr. Then a standard drop of 4 per cent washed sheep erythrocytes sensitized with 4 HD_{50} of haemolysin was added and the trays were mechanically shaken for 2 hours at 35°. The degree of haemolysis was estimated after the cells had settled, 50 per cent lysis being taken as the endpoint.

EXPERIMENTAL

GENERAL EXPERIMENTAL DESIGN

Antigens

The dose was the same for each antigen and the ratio of dose of haemagglutinin to number of electron microscopically visible particles is similar for the three antigens (Fazekas de St. Groth and Webster, 1963). Therefore the number of particles and the amount of antigen in each dose would have been nearly constant.

Routes of vaccination

The immunizing dose, always contained in 0·25 ml of standard medium, was inoculated either intravenously into the marginal vein of the ear or intraperitoneally through the lateral side of the rectus abdominis muscle or subcutaneously over the lumbar region. Water-in-oil-emulsions of the antigens were injected intramuscularly into the gluteus muscles.

TABLE 1

GROUPING OF RABBITS FOR VACCINATION

Schedule of administration	Inocula								
	Intravenous			Intraperitoneal			Subcutaneous		
	Initial	Secondary	Tertiary	Initial	Secondary	Tertiary	Initial	Secondary	Tertiary
Group I (single-aqueous)	0	40	60	0	40	60	0	40	60
Group II (multiple-aqueous)	0, 2, 4, 7, 9, 11	40, 42, 44	60, 62, 64	0, 2, 4, 7, 9, 11	40, 42, 44	60, 62, 64	0, 2, 4, 7, 9, 11	40, 42, 44	60, 62, 64
Group III (single-adjuvant)	0*	40	60	0*	40	60	0*	40	60
Group IV (multiple-adjuvant)	0*, 2, 4, 7, 9, 11	40, 42, 44	60, 62, 64	0*, 2, 4, 7, 9, 11	40, 42, 44	60, 62, 64	0*, 2, 4, 7, 9, 11	40, 42, 44	60, 62, 64

The figures show the days on which the three rabbits of a group received 5000 haemagglutinating doses of either MEL, LEE or SW influenza virus.

*Received antigen in Freund's incomplete adjuvant (Difco) by the intramuscular route.

Schedules of inoculation

Schedules of inoculation are set out in Table 1. Rabbits in Group I received a single dose of antigen without adjuvant; Group II received multiple doses of antigen without adjuvant; Group III received a single dose of antigen in adjuvant and Group IV received multiple doses of antigen, the first dose being with adjuvant.

Timing

In this paper the term initial as applied to antigen injection(s) or serological response means events occurring within the first 39 days after the first injection of antigen, 'secondary' applies to the interval 40–59 days and 'tertiary' applies to day 60 and thereafter.

Titration of antibody

Sera were stored at −15° and tested at the end of an experiment by the three methods described above. In each method titrations were expressed in terms of 1·0 ml volumes. The results of neutralization tests were transformed into neutralizing potencies or pN (Fazekas de St. Groth, 1961).

PRELIMINARY EXPERIMENTS

Stability of reagents

Before the results obtained in a study extending over several years can be legitimately compared, it is necessary to demonstrate that the reagents remained unchanged over the period of observations.

Antisera are usually assumed to be stable when stored in the frozen state. In order to furnish formal evidence on this point a set of sera, one against each of the three strains of influenza virus used in these studies, was tested for neutralizing potency over a span of 2 years (Table 2).

Table 2

The stability of antibody: the neutralizing potencies of three sera on storage, with freezing and thawing

Antisera	Neutralizing potency	
	1960	1962
Anti-LEE	4·51±0·04*	4·58±0·03
Anti-SW	4·79±0·04	4·85±0·03
Anti-MEL	4·31±0·06	4·27±0·06

*Standard error.

Evidently, storage at −15°, even with frequent thawing and refreezing, did not alter the neutralizing potencies of the antisera. Similar control tests, using antihaemagglutinin or complement fixation tests as methods of assay, gave the same answer.

The infectivity of influenza virus preparations stored at −70° does not change over a period of 2 years as has been demonstrated elsewhere, using the same preparations of virus as in the present study (Fazekas de St. Groth and Webster, 1963). The haemagglutinin of influenza virus eluates stored at 4° with 0·08 per cent sodium azide as preservative has been tested over a similar period and, provided the virus was stored in detergent-free glassware, the haemagglutinin titres remained unchanged over at least 2 years.

The response to live and killed influenza vaccines

Four groups of two rabbits each were vaccinated by the intravenous route with 5000 haemagglutinating doses of SW influenza virus. The first group received infective virus eluate, the second heat inactivated virus, the third ultraviolet treated virus and the fourth virus treated with both formaldehyde and ultraviolet radiation. All rabbits were bled

prior to vaccination and at intervals thereafter, and the sera were tested for antihaemagglutinin after the conclusion of the experiment. The results are presented in Fig. 1. The antibody responses are seen to be virtually identical in all four groups. Accordingly, live virus eluates were administered in all following experiments as immunizing antigens, without any further treatment.

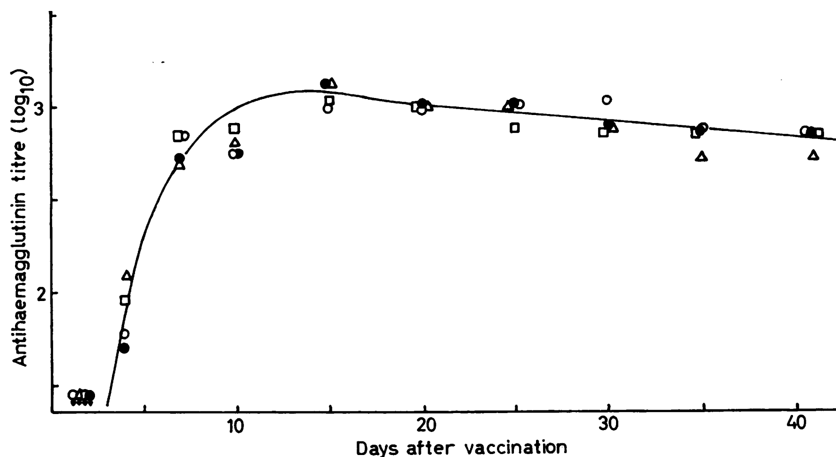

FIG. 1. The antibody response of the rabbit to live and killed influenza vaccines. Each point represents the geometric mean titre of a group of two rabbits.

●, Active virus; □, heat-inactivated virus; △, UV-inactivated virus; ○, UV and formaldehyde inactivated virus.

MAIN EXPERIMENTS

Each of the experiments of which the results are shown in the figures was performed on thirty-six rabbits, three animals to each of the twelve treatments. After completion of a course of injections and bleedings the sera were assayed in orthogonal groups, always in multiples of twelve. Neutralization tests were usually done in sets of seventy-two per day. The inherent error of a single titration is $\pm 0{\cdot}118$ (Fazekas de St. Groth *et al.*, 1958). For determination of the maximum levels of serum antibody three or four successive bleedings were used and the average of these was found to carry standard deviations of $\pm 0{\cdot}246$ for MEL, $\pm 0{\cdot}267$ for LEE and $\pm 0{\cdot}335$ for SW virus and their respective antibodies. The rates of antibody production were estimated in a digital computer by fitting straight lines to the first three or four points on the rising sections of each response curve, with an

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FIG. 2. The antibody response of rabbits to a type A influenza virus (MEL), as measured by neutralizing potency, complement fixation and antihaemagglutination. Antibody titres are expressed in log_{10} units per 1·0 ml volumes of serum. Each point represents the geometric mean titre of a group of three rabbits.

Schedules of vaccination:

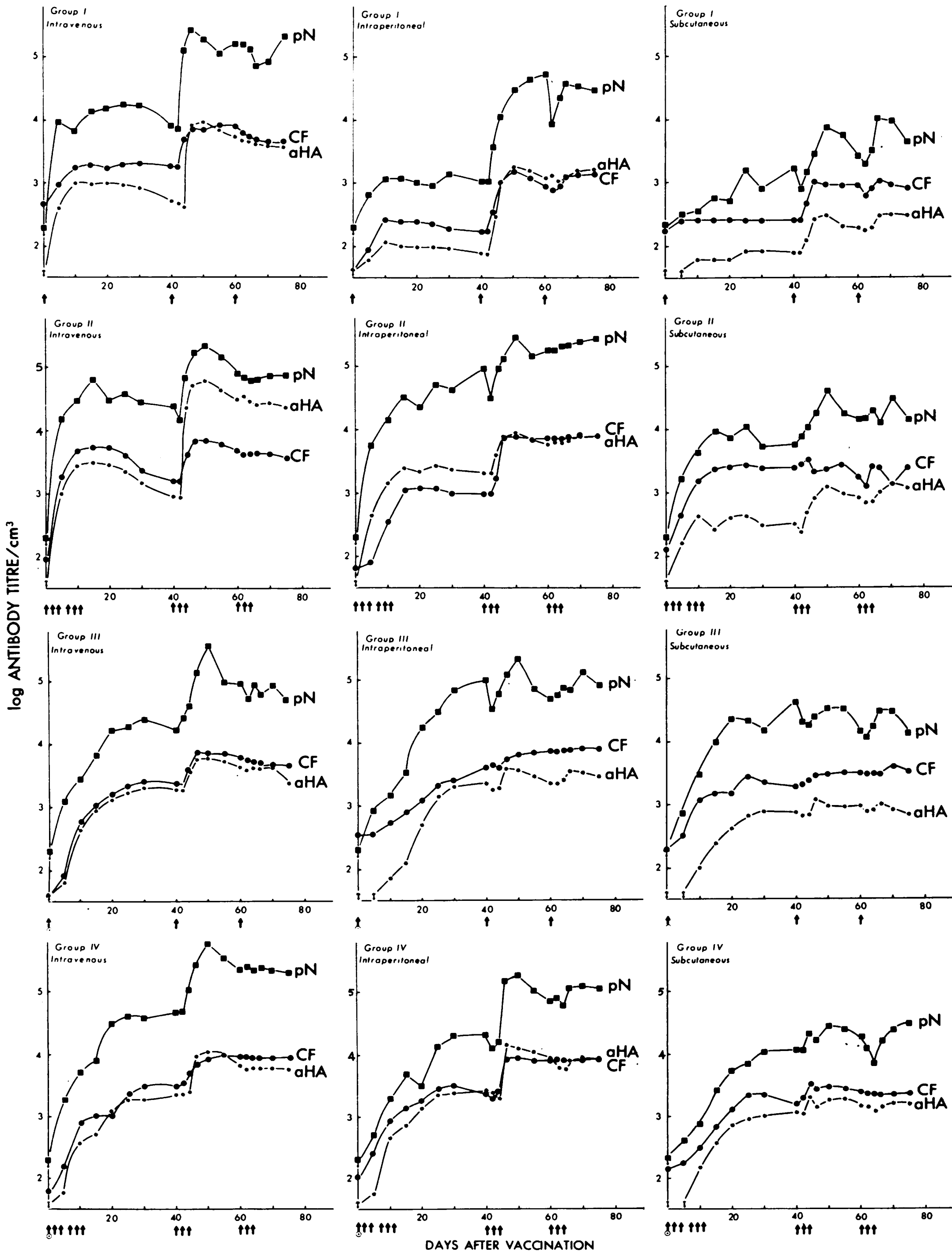
Group I: Aqueous vaccine on days 0, 40 and 60, by the route indicated on the figures.

Group II: Aqueous vaccine on days 0, 2, 4, 7, 9, 11 as initial stimulus, on days 40, 42, 44 as secondary and on days 60, 62, 64 as tertiary stimulus.

Group III: Vaccine in incomplete Freund's adjuvant intramuscularly on day 0, aqueous vaccine on days 40 and 60, by the routes indicated on the figures.

Group IV: Vaccine in incomplete Freund's adjuvant intramuscularly on day 0, followed by aqueous vaccine on days 2, 4, 7, 9 and 11 as initial stimulus. by the routes indicated on the figures. The secondary and tertiary courses of vaccination were the same as for the corresponding sub-sets of Group II.

Each inoculum of vaccine contained 5000 haemagglutinating units of virus (approximately 10^{11} particles, or 15 μg).


Group I
Intravenous
Group I
Intraperitoneal
Group I
Subcutaneous
Group II
Intravenous
Group II
Intraperitoneal
Group II
Subcutaneous
Group III
Intravenous
Group III
Intraperitoneal
Group III
Subcutaneous
Group IV
Intravenous
Group IV
Intraperitoneal
Group IV
Subcutaneous
pN
CF
aHA
log ANTIBODY TITRE/cm³
DAYS AFTER VACCINATION

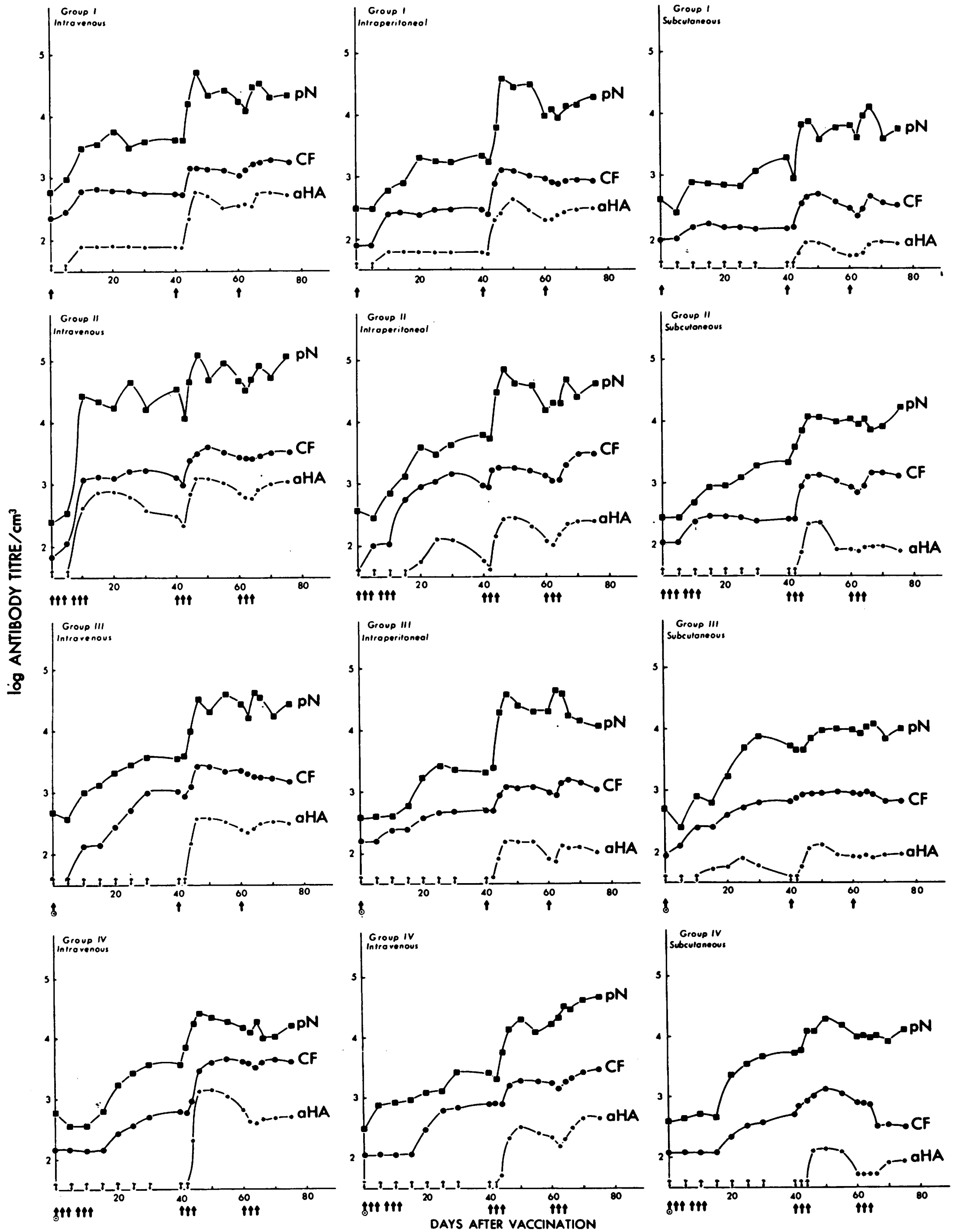


FIG. 3. The antibody response of rabbits to a type B influenza virus (LEE), as measured by neutralizing potency, complement fixation and antihaemagglutination. (Schedules of vaccination as for Fig. 2.)

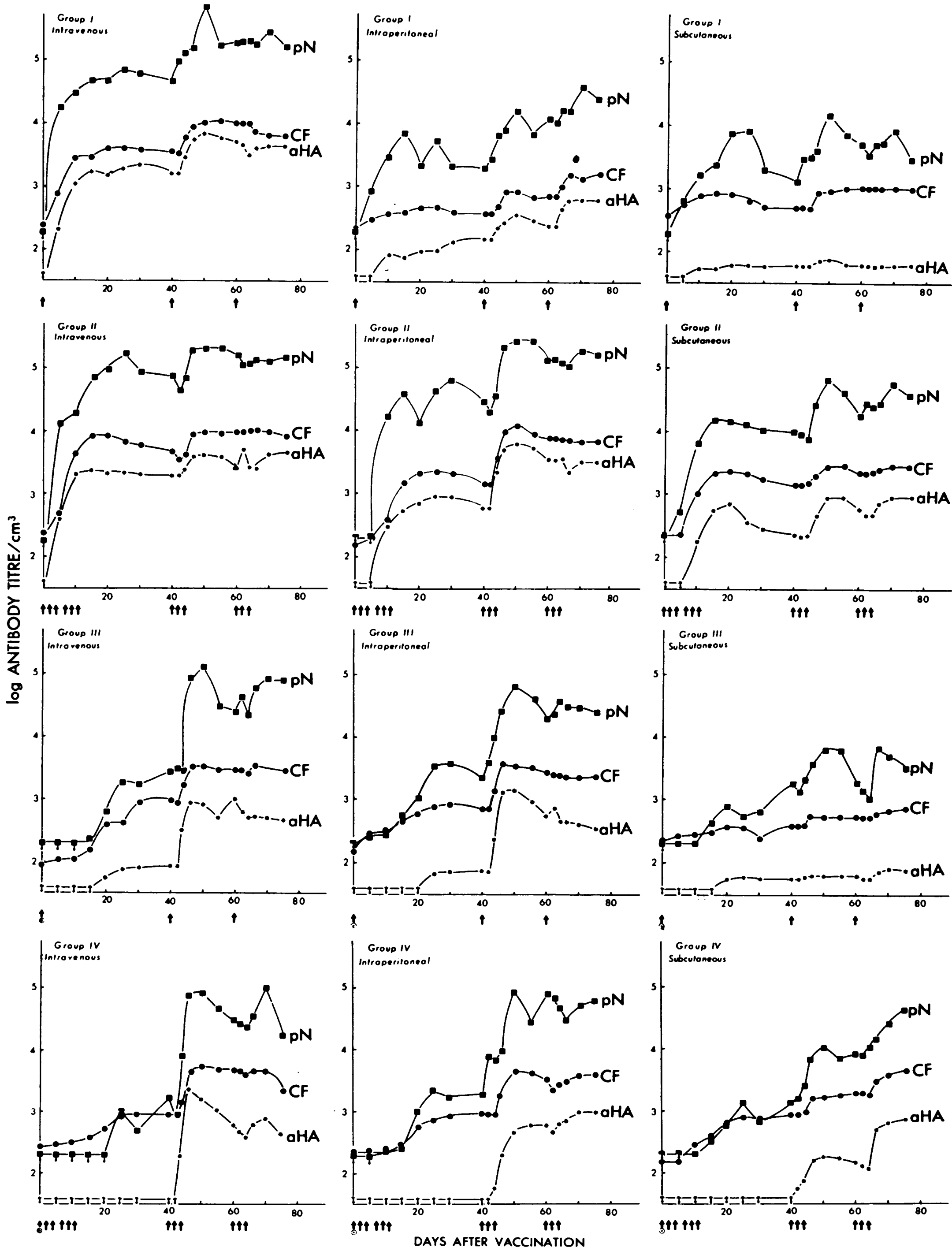


FIG. 4. The antibody response of rabbits to swine influenza virus (SW), as measured by neutralizing potency, complement fixation and antihaemagglutination. (Schedules of vaccination as for Fig. 2.)

accuracy of $\pm 0{\cdot}010$ for MEL, $\pm 0{\cdot}007$ for LEE and $\pm 0{\cdot}011$ for SW, in $\log_{10}$ units per day, i.e. an uncertainty of $\pm 3{\cdot}3$, $\pm 2{\cdot}3$ and $\pm 3{\cdot}7$ per cent in the estimated doubling times. Antihaemagglutinin tests have an inherent error of $\pm 0{\cdot}063$ $\log_{10}$ units in a single titration (Fazekas de St. Groth and Webster, 1961). One unit of antibody is taken as the amount that prevents three out of four HA doses from agglutinating chicken red blood cells in the antihaemagglutinin test, prevents 50 per cent lysis in the complement fixation test, and 50 per cent infection in the neutralization test. The units are expressed in $\log_{10}$ terms. The titration of maximal levels of serum antibody had a standard deviation of $\pm 0{\cdot}080$ for MEL, $\pm 0{\cdot}088$ for LEE and $\pm 0{\cdot}116$ for SW and their respective antibodies. The accuracy of measurement of the rates of antibody production by antihaemagglutination was $\pm 0{\cdot}002$ for MEL, $\pm 0{\cdot}005$ for LEE and $\pm 0{\cdot}007$ for SW in $\log_{10}$ units per day.

Both these estimations and the comparison of starting times, rates of production and maximum levels attained were carried out by programming a digital computer to perform the required analyses of variance and covariance, and to compute all the means and standard errors to appear in this paper. The analyses were non-orthogonal due to serial correlation of the initial, secondary and tertiary antibody responses (which were measured on the same animals) and due to some missing entries (six out of 108 animals died by misadventure during the course of the experiments). The nonorthogonality has been allowed for when computing variances and covariances.

The immune response to the twelve test-treatments is given separately for the three strains of virus used as antigens. Each point represents the geometric mean titre of a group of three rabbits; the curves were fitted by eye. The results of the statistical calculations are summarized in Tables 3, 4 and 5.

TABLE 3
LAG PHASE PRECEDING THE APPEARANCE OF ANTIBODY

Vaccine strain of virus	Schedule of administration	Route of inoculation								
		Intravenous			Intraperitoneal			Subcutaneous		
		Neut	C.F.	aHA	Neut	C.F.	aHA	Neut	C.F.	aHA
MEL	Group I	2·34	2	2	2·99	2	2	4·87	4	6
	Group II	2·38	2	2	1·69	2	2	2·30	2	2
	Group III	2·42	2	3	2·70	6	6	2·96	3	6
	Group IV	1·28	2	3	3·30	2	3	2·73	3	6
LEE	Group I	2·76	3	6	4·20	6	6	2·50	6	>40
	Group II	2·60	3	6	5·20	3	16	2·90	6	>40
	Group III	4·60	6	>40	5·60	6	>40	9·40	3	11
	Group IV	16·70	16	>40	5·70	16	>40	13·60	16	>40
SW	Group I	2·31	2	2	3·73	3	6	3·10	3	6
	Group II	2·44	2	2	5·00	3	6	3·10	6	6
	Group III	17·30	11	11	9·80	3	21	14·00	5(?)	16
	Group IV	19·50	5(?)	>40	12·10	10	>40	15·00	6(?)	>40

The figures show the time in days between injection and first detection of antibody.
Group I (single aqueous); Group II (multiple aqueous); Group III (single adjuvant); Group IV (multiple adjuvant).
Neut = Neutralization; C.F. = Complement fixation; aHA = Antihaemagglutination.

RESULTS AND CONCLUSIONS

From all analyses of variance and covariance the differences between the immune responses of different rabbits emerged as a factor significantly contributing to the overall

TABLE 4

THE RATE OF INCREASE IN LEVELS OF SERUM ANTIBODY

Vaccine strain of virus	Schedule of administration	Initial response									Secondary response									Tertiary response								
		Intravenous			Intraperitoneal			Subcutaneous			Intravenous			Intraperitoneal			Subcutaneous			Intravenous			Intraperitoneal			Subcutaneous		
		Neut	C.F.	aHA	Neut	C.F.	aHA	Neut	C.F.	aHA	Neut	C.F.	aHA	Neut	C.F.	aHA	Neut	C.F.	aHA	Neut	C.F.	aHA	Neut	C.F.	aHA	Neut	C.F.	aHA
MEL	Group I	0·66	0·06	0·17	0·12	0·07	0·06	0·07	0·07	0·01	0·39	0·15	0·31	0·26	0·20	0·34	0·14	0·15	0·16	0·00	0·00	0·00	0·15	0·00	0·07	0·18	0·06	0·10
	Group II	0·73	0·17	0·22	0·25	0·12	0·19	0·19	0·11	0·10	0·27	0·17	0·20	0·25	0·23	0·14	0·10	0·01	0·11	0·00	0·00	0·00	0·02	0·01	0·01	0·99	0·07	0·05
	Group III	0·17	0·15	0·12	0·14	0·13	0·12	0·18	0·05	0·09	0·18	0·12	0·12	0·11	0·31	0·09	0·02	0·05	0·06	0·00	0·00	0·00	0·06	0·00	0·05	0·10	0·00	0·02
	Group IV	0·20	0·09	0·11	0·16	0·10	0·13	0·04	0·06	0·11	0·19	0·08	0·17	0·28	0·17	0·20	0·07	0·04	0·02	0·00	0·00	0·00	0·04	0·00	0·03	0·09	0·00	0·00
LEE	Group I	0·07	0·01	0·03	0·05	0·05	0·02	0·05	0·03	0·00	0·27	0·24	0·23	0·34	0·18	0·15	0·23	0·12	0·08	0·15	0·05	0·05	0·03	0·02	0·03	0·13	0·10	0·03
	Group II	0·20	0·10	0·08	0·07	0·08	0·02	0·05	0·05	0·00	0·26	0·13	0·20	0·28	0·10	0·21	0·12	0·18	0·10	0·10	0·01	0·03	0·09	0·07	0·09	0·00	0·08	0·02
	Group III	0·05	0·04	0·00	0·06	0·02	0·00	0·09	0·03	0·02	0·22	0·16	0·25	0·31	0·10	0·15	0·04	0·01	0·05	0·08	0·01	0·04	0·07	0·06	0·05	0·12	0·00	0·00
	Group IV	0·02	0·03	0·00	0·03	0·04	0·00	0·09	0·03	0·00	0·26	0·17	0·38	0·21	0·09	0·29	0·07	0·06	0·25	0·00	0·01	0·01	0·07	0·05	0·16	0·00	0·03	0·01
SW	Group I	0·76	0·11	0·19	0·18	0·03	0·01	0·15	0·03	0·02	0·02	0·11	0·14	0·12	0·09	0·07	0·06	0·03	0·04	0·00	0·00	0·00	0·03	0·09	0·10	0·00	0·00	0·00
	Group II	0·70	0·14	0·20	0·26	0·09	0·11	0·22	0·10	0·12	0·08	0·10	0·08	0·25	0·21	0·15	0·16	0·04	0·10	0·01	0·01	0·00	0·00	0·00	0·00	0·01	0·01	0·05
	Group III	0·08	0·06	0·01	0·07	0·03	0·02	0·07	0·10	0·02	0·36	0·15	0·26	0·21	0·18	0·32	0·13	0·04	0·01	0·04	0·01	0·05	0·02	0·00	0·00	0·16	0·00	0·04
	Group IV	0·09	0·03	0·00	0·07	0·01	0·00	0·06	0·03	0·00	0·39	0·18	0·36	0·02	0·11	0·10	0·17	0·07	0·11	0·03	0·00	0·02	0·00	0·03	0·05	0·07	0·05	0·16

The figures show the initial slopes ($\log_{10}$ rise/day) of the respective response curves, based on the geometric mean antibody titres from groups of three rabbits. The schedules of vaccination are set out in Table 1.

Group I (single aqueous); Group II (multiple aqueous); Group III (single adjuvant); Group IV (multiple adjuvant).

Neut. = Neutralization; C.F. = Complement fixation; aHA = Antihaemagglutination.

TABLE 5

MAXIMUM LEVELS OF SERUM ANTIBODY

Vaccine strain of virus	Schedule of administration	Primary response									Secondary response									Tertiary response								
		Intravenous			Intraperitoneal			Subcutaneous			Intravenous			Intraperitoneal			Subcutaneous			Intravenous			Intraperitoneal			Subcutaneous		
		Neut	C.F.	aHA	Neut	C.F.	aHA	Neut	C.F.	aHA	Neut	C.F.	aHA	Neut	C.F.	aHA	Neut	C.F.	aHA	Neut	C.F.	aHA	Neut	C.F.	aHA	Neut	C.F.	aHA
MEL	Group I	4·22	3·30	3·02	3·07	2·50	2·10	3·13	2·40	1·91	5·29	3·90	3·43	4·30	3·25	3·20	3·81	2·45	2·44	5·06	3·70	3·59	4·51	3·13	3·13	3·99	3·13	2·49
	Group II	4·63	3·72	3·50	4·79	3·02	3·45	3·91	3·48	2·58	5·26	3·82	3·74	5·30	3·88	3·89	4·37	3·35	2·85	4·85	3·60	3·41	5·39	3·89	3·87	4·50	3·39	2·87
	Group III	4·30	3·38	3·32	4·90	3·51	3·32	4·38	3·41	2·86	5·11	3·90	3·78	5·08	3·81	3·60	4·47	3·49	3·02	4·79	3·69	3·64	4·95	3·84	3·59	4·47	3·40	2·97
	Group IV	4·63	3·50	3·40	4·25	3·45	3·35	4·05	3·32	3·02	5·59	3·95	4·01	5·26	3·93	4·10	4·44	3·48	3·27	5·33	3·96	3·76	5·05	3·91	3·85	4·39	3·37	3·16
LEE	Group I	3·59	2·79	1·93	3·27	2·54	1·83	3·07	2·17	<1·60	4·48	3·15	2·65	4·61	3·05	2·59	3·69	2·65	1·90	4·42	3·25	2·73	4·22	2·93	2·45	3·82	2·64	1·88
	Group II	4·42	3·18	2·74	3·63	3·09	2·04	3·15	2·45	<1·60	4·92	3·55	3·06	4·73	3·23	2·46	4·04	3·08	2·32	4·80	3·52	2·98	4·54	3·50	2·37	4·01	3·14	1·95
	Group III	3·75	2·67	<1·60	3·34	2·76	<1·60	3·74	2·80	1·75	4·46	3·36	2·58	4·39	3·09	2·19	3·89	2·96	2·08	4·31	3·23	2·53	4·14	3·14	2·06	3·96	2·86	1·94
	Group IV	3·52	2·73	<1·60	3·30	2·84	<1·60	3·63	2·68	<1·60	4·84	3·60	3·08	4·16	3·29	2·50	4·17	3·05	2·10	4·55	3·61	2·66	4·52	3·44	2·63	3·97	2·97	1·76
SW	Group I	4·78	3·61	3·04	3·60	2·67	2·18	3·88	2·73	1·91	5·26	4·00	3·82	3·99	2·41	2·52	3·75	2·95	1·99	5·31	3·83	3·64	4·41	3·26	2·80	3·75	3·12	1·92
	Group II	5·06	3·89	3·34	4·51	3·33	2·92	4·07	3·39	2·73	5·31	3·96	3·60	5·39	4·00	3·76	4·54	3·17	2·89	5·13	4·00	3·68	5·18	3·83	3·45	4·56	3·42	2·94
	Group III	3·20	2·90	1·90	3·49	2·88	1·82	2·87	2·60	1·75	5·00	3·50	2·85	4·60	3·47	3·11	3·72	2·70	1·80	4·85	3·49	2·68	4·44	3·35	2·62	3·68	2·90	1·89
	Group IV	2·79	2·94	<1·60	3·31	2·95	<1·60	2·95	2·93	<1·60	4·83	5·69	3·13	4·45	3·55	2·86	3·89	3·23	2·22	4·60	3·57	2·79	4·68	3·55	2·96	4·40	3·61	2·80

The figures show the mean antibody titres ($\log_{10}$ titre/ml) of groups of three rabbits, expressed in terms of 1·0 ml volumes, averaged for at least three determinations over 10 days during the peak of antibody production. The schedules of vaccination are set out in Table 1.

Group I (single aqueous); Group II (multiple aqueous); Group III (single adjuvant); Group IV (multiple adjuvant).

Neut = Neutralization; C.F. = Complement fixation; aHA = Antihaemagglutination.

variation. Since the genetic factors contributing to this variation could not have been studied effectively in groups of the size used in the experiments and in animals bred without any selection, this factor was eliminated from all comparisons, and the error sum of squares was increased by all interaction sums of squares containing differences between rabbits as factors. As a result, the error variances were increased by a factor of 2·33 in the evaluation of the levels of antibody production, by 1·24 in the comparison of starting times, and by 1·42 in comparing the rates of increase in neutralizing antibody. The increments of the error variance with antihaemagglutinin tests were 13·59- and 1·40-fold respectively for levels and rates and with complement fixation tests they were 10·53- and 1·86-fold for levels and rates. These factors show also that the rabbits differed considerably in the maximum levels of serum antibody, less so in the rate of production, and hardly at all in the lag period preceding the appearance of newly formed antibody. It should be added that all of the significant variance ratios due to the main contrasts remained significant at the 1 per cent level even after the error variances had been increased.

All titrations for neutralizing antibody were started at serum dilutions of 1 : 3·16. At this level all normal sera failed to neutralize MEL and SW antigens, but normal sera did neutralize LEE virus, with titres of 1 : 5 to 1 : 20. Thus the primary response against LEE could only be followed over a narrower range than was the case with the other two antigens. The presence of this background neutralization would not influence estimates of the maximum antibody levels attained, but could obscure the true starting times and cause the rate of increase of early antibodies to appear less than it was.

Complement fixation and antihaemagglutinin titrations were both started at serum dilutions of 1 : 20. At this dilution no non-specific inhibition was detected in the antihaemagglutinin test but many of the pre-vaccination sera fixed complement in the presence of influenza virus antigens, some to titres of 1 : 100. This non-specific effect did not influence estimates of the maximal antibody levels but did mask the true starting times and may have decreased the slope of the response curve. For this reason the starting times of antibody formation were read from the figures.

THE LAG PHASE

The lag phase preceding the appearance of antibody has been considered as the interval between initial injection and the time of first detection of antibody. Because of the above mentioned non-specific effects accurate estimations of the lag phase were possible only with MEL and SW antigens and then only in neutralization tests.

In general it seemed that the length of the lag phase was determined by the first injection and was not affected by subsequent injections. In some cases (e.g. the antihaemagglutinin results from LEE vaccine given in a single aqueous injection by the subcutaneous route) the figure >40 indicates that the specific response did not rise above the basic non-specific level during the course of the whole period of 40 days. In some cases, particularly in those groups that received subsequent doses of antigen at two daily intervals after the initial dose of antigen in adjuvant by the intramuscular route, interference by non-specific inhibitor may not have been the complete answer, and this is discussed later.

THE RATES OF INCREASE IN LEVELS OF SERUM ANTIBODY

Rates for the various schedules and antigens are presented in Table 4, in terms of $\log_{10}$ units per day. As an example 0·66 $\log_{10}$ units per day represents a doubling time of 0·46 days, 0·12 units a doubling time of 2·5 days.

Comparison of the rates obtained by the three serological procedures indicated that the sensitivity of the test employed greatly influenced the estimations of rate. The neutralization test was more sensitive than the antihaemagglutinin test which was slightly more sensitive than the complement fixation test. The rates of antibody formation after aqueous vaccination by the intravenous route were in general higher than after vaccination by the intraperitoneal or subcutaneous route. Multiple inoculations of aqueous vaccine gave higher rates than single doses. Single inoculations of adjuvant vaccine, gave very low rates of antibody formation with LEE and SW antigens, and these levels were further depressed by multiple doses of aqueous vaccine.

The most striking feature observed when the three tests were compared for the initial response period was that the very high rates of antibody production as measured by the neutralization test after intravenous inoculation of aqueous vaccine were not observed with the antihaemagglutinin or complement fixation tests.

This difference was observed only after single or multiple doses of vaccine by the intravenous route and was more pronounced with MEL and SW vaccines than with LEE. Inoculation of aqueous vaccine by the intraperitoneal or subcutaneous route, or of any of the antigens in adjuvant by the intramuscular route did not give results where the disparity could not be explained simply by the sensitivity of the test. The example of multiple aqueous doses of MEL vaccine by the intraperitoneal route served to illustrate the point; under these conditions the doubling time for antibody as measured by neutralization was 1·2 days, by complement fixation 2·5 days and by antihaemagglutination 1·6 days.

The second striking feature was that in many instances after vaccination with LEE or SW antigens in adjuvant, antibody could not be detected with the antihaemagglutinin test.

The values for the rates of serum antibody production after secondary stimulation may be underestimates because straight lines fitted to the points extrapolated to points in time preceding the injections. The initial rise was either masked by antibody from earlier stimulation or when measurements became possible, the rate of elimination was not negligible as compared with the rate of production.

A comparison of the initial and secondary rates showed that the secondary rates were uniformly higher than the initial and showed little variation with treatment, and there was no disparity between tests. A typical example from Table 4 would be the doubling time of antibody after LEE vaccine had been inoculated intraperitoneally in aqueous suspension. As measured by neutralization the doubling time was 0·9 days, by complement fixation 1·7 days and by antihaemagglutination 2·0 days.

The lack of significant rises in antibody titres after tertiary stimulation is apparent from Table 4. Many of the treatment groups showed no increase and those that did, gave doubling times of from 3 to 10 days. There was no correlation with routes, schedules, or antigens and it will become apparent later that the tertiary responses served only to return the level of antibody to that obtained in the secondary response.

MAXIMUM LEVELS OF SERUM ANTIBODY

Maximal levels of serum antibody for the various treatment groups are presented in Table 5 in $\log_{10}$ units per millilitre. In the initial response period there was a significant difference between routes of inoculation; the intravenous route was superior to the intraperitoneal and this in turn was superior to the subcutaneous route. Multiple doses of antigen in aqueous suspension given by any of the three routes yielded higher antibody

titres than did single doses of antigen. The intravenous route was superior to the intraperitoneal or subcutaneous.

Vaccines administered in adjuvant did not produce higher levels of antibody than did aqueous vaccines in the initial response. In fact there was a marked depression of antibody production with LEE and SW antigens, which was further accentuated when multiple doses of aqueous vaccine were given after the initial dose of antigen in adjuvant.

In several treatment groups the anamnestic response was preceded by a negative phase; the sera taken 2 days after the boosting inoculum contained lower titres of antibody than the sera taken immediately before boosting. The fall in antibody titres was not observed regularly, and varied even within a single group, i.e. among rabbits receiving identical treatments. Thermodynamic measurements indicate that the drop in titre is due to binding of antibody to the injected antigen; the most avid antibody is bound and accounts for the decrease in titre.

Secondary stimulation with antigen in aqueous suspension caused a marked rise in maximal antibody titres by all routes of inoculation; all antigens induced antibody titres that were from 8- to 10-fold above the maximal initial antibody titres. Single boosting doses of the three antigens gave higher titres by the intravenous route than by the intraperitoneal or subcutaneous routes. Multiple doses of antigen in aqueous suspension eliminated this difference and comparable titres were obtained by the intravenous and intraperitoneal routes, while the subcutaneous route gave lower titres.

Single doses of booster antigen in aqueous suspension were in general as effective as multiple doses in eliciting maximal levels of antibody after initial stimulation in adjuvant. The route of inoculation was however important, the intravenous and intraperitoneal routes were superior to the subcutaneous route.

Tertiary stimulation of all treatment groups served only to return the maximal titres of antibody to those obtained in the secondary response but did not stimulate higher levels of antibody even in the groups that received vaccine by the subcutaneous route. One was forced to conclude that under these conditions the animals had reached the maximal titres of antibody that they could attain.

Comparisons of the maximal antibody titres as measured by the three serological tests are presented in Table 6.

TABLE 6

COMPARISON OF THE RATIOS OF MAXIMAL ANTIBODY TITRES

Antigen	Ratio	Initial response				Secondary response			
		Group I	Group II	Group III	Group IV	Group I	Group II	Group III	Group IV
MEL	Neut/aHA	1·16	1·26	1·36	1·09	1·28	1·49	1·42	1·31
	Neut/C.F.	0·74	1·03	1·10	0·89	1·10	1·20	1·16	1·31
	C.F./aHA	0·42	0·33	0·26	0·20	0·18	0·19	0·26	0·00
LEE*	Neut/aHA	1·68	1·64	1·83	1·98	1·88	1·95	1·97	1·83
	Neut/C.F.	0·93	0·82	0·67	0·73	1·31	1·33	1·11	1·08
	C.F./aHA	0·75	0·82	1·16	1·25	0·57	0·68	0·86	0·65
SW	Neut/aHA	1·71	1·55	1·37	1·52*	1·55	1·66	1·85	1·65
	Neut/C.F.	1·09	1·02	0·40	0·08*	1·05	1·37	1·22	0·90
	C.F./aHA	0·62	0·53	0·97	1·44*	0·50	0·29	0·63	0·75

*In the initial response, ratios for LEE antigen in all groups, and for SW antigen in Group IV are inaccurate owing to antibody levels of some animals being below baseline.

Figures show the ratios in $\log_{10}$ units between the maximal levels of antibody in groups of nine rabbits.

Neut = Neutralization; C.F. = Complement fixation; aHA = Antihaemagglutination.

The ratios of the mean maximal antibody titres as measured by neutralization and antihaemagglutination were constant within the initial and secondary responses, but were significantly different between the responses. The ratios were independent of the route and schedule of inoculation but differed with the strain of antigen. This was also true for the ratios between the results of neutralization and complement fixation tests, but in those groups that responded poorly after stimulation with antigen in adjuvant there were differences at the 0·1 per cent level of significance. In these cases the complement fixation titres approached the neutralizing titres suggesting that antibody of poor avidity was being produced in moderate quantity. The ratios of complement fixation to antihaemagglutination carried much smaller errors and were not constant. The adjuvant groups were distinctly different from the groups receiving aqueous vaccines although they were generally constant within the groups. Each of the three ratios was different for the three strains of antigen in the initial response, but the neutralization–complement fixation ratios were not significantly different in the secondary response.

The differences in antibody levels were most clearly seen after secondary stimulation, as here they could not be attributed to, or confused by the sensitivity of the tests. After secondary stimulation the overall increase in titre as measured by neutralization was 6·3-fold, by antihaemagglutination 4·2-fold and by complement fixation 2·5-fold. The extent of this increase varied with the route and schedule of vaccination, but not with antigen.

This disproportionate increase in antibody titres as detected by the three serological methods after secondary stimulation was particularly marked in the treatment groups that received an initial dose of antigen in adjuvant, followed by multiple doses of antigen in aqueous suspension. This was particularly evident with SW and LEE antigens that gave low levels of antibody in the initial response period. Under these conditions for SW antigen the neutralization test measured a 23·4-fold increase in antibody titre after secondary stimulation while the complement fixation test measured only a 3·5-fold increase. The route of inoculation also influenced the different increases in titre as measured by the three tests, intravenous inoculation caused greater differences than did intraperitoneal inoculation, which in turn was more potent than subcutaneous inoculation. Secondary stimulation clearly caused some change in the antibody population detected by the three serological tests. As the complement fixation test measures predominantly the quantity of antibodies and the neutralization test the avidity of antibodies, one explanation of the disproportionate increase would be an increase in the binding capacity of antibody. A second explanation could be that in the initial response period, a proportion of the antibody population (γ_2-19S antibody) fixes complement more efficiently (Taliaferro and Taliaferro, 1961; Nussenzweig, Merryman and Benacerraf, 1964), and that this fraction of the antibody population was reduced after secondary stimulation.

Both of these explanations for the disproportionate increase in antibody levels by the three serological tests are probably valid. Preliminary thermodynamic observations showed that the avidity of antibody did increase on secondary stimulation, and that there was a higher concentration of 19S type antibody in the initial than in the secondary response period.

DISCUSSION

As well as providing practical answers and establishing a reference system for studying the immune response to influenza virus the aim of the present study was to determine if by the use of three serological tests changes in avidity of antibody could be detected.

Complement fixation tests measure essentially the quantity of antibody; antihaemagglutinin tests measure both quantity and avidity while neutralization tests measure essentially avidity (Fazekas de St. Groth, 1962). It should be expected therefore that the ratio of antibody titres obtained by these three techniques would change whenever the avidity of antibody changed, while the absolute magnitude of the titres would be more closely related to the quantity of antibody in the test samples, although not linearly.

The results show that the greatest change in the ratios occurred between the initial and secondary responses. These changes and the other observations made in this study are listed below, and an attempt is made to fit them into the current theories of antibody production.

1. The shortness of the inductive phase after primary stimulation with aqueous suspensions of virus;
2. The high rates of primary antibody formation as detected by neutralization tests after intravenous administration of antigen.
3. The depressive action of incomplete water-in-oil adjuvants, on antibody production;
4. The change in ratios of the serological tests between the initial and secondary response.
5. The attainment of different maximal levels of antibody, depending on the route and schedule of vaccination, levels which were not surpassed after tertiary stimulation.

The current theories of antibody production fall into two categories, the instructive theory (Breinl and Haurowitz, 1930; Alexander, 1931; Mudd, 1932; Pauling, 1940) and the selective theory (Ehrlich, 1900; Jerne, 1955; Talmage, 1957; Burnet, 1957; Lederberg, 1959). The basic question is whether the information for specific antibody is brought into the cell by the antigen (instructive) or is inherent in the cell waiting to be evoked (selective). The first theory implies that the cells required for antibody formation are already present in sufficient numbers, the second that a clone of cells develops as a result of antigenic stimulation.

The early appearance of antibody after initial stimulation can be accommodated by either theory, but the dependence of rates of antibody formation on the route of inoculation and on the test used is difficult to explain by either theory. A possible improvement in the avidity of antibody during the earliest phase of production could be invoked to account for the observations, but since directional variation of the kind of antibody produced is contrary to expectations from either hypothesis, such an interpretation would equally weaken both. The recent observations on the changes in the physical properties of antibody during the primary antibody response (Uhr and Finkelstein, 1963; Svehag and Mandel, 1964) and the bimodal nature of the primary response to influenza virus (Fazekas de St. Groth and Webster, 1964) emphasizes facts that must be accommodated in any comprehensive theory of antibody production.

The depressive action of adjuvant on the starting times, rates and maximal levels of primary antibody production, especially with SW and LEE antigens, cannot be fitted into either theory. The slow release of antigen from the adjuvant depot could be used as an explanation in the treatment-groups receiving single doses of antigen in water-in-oil suspensions. However, this could not be said for the companion group, where the intramuscular dose of adjuvant vaccine was followed by five doses of antigen in aqueous suspension at two-daily intervals.

The findings of Paraf, Fougerau and Bussard (1963) were similar to the observations on adjuvant vaccination. They found that water-in-oil adjuvants, even when given at

separate sites, or preceding the immunizing antigen by several days, depressed the production of antibody. The phenomenon depends on the dosage of antigen and adjuvant as well as on the timing of the two injections. The results reported above indicated that it also depends on the nature of the antigen, but has nothing to do with its inherent immunogenicity. The latter follows from the fact that SW virus is almost as good an antigen as MEL, while LEE is considerably poorer than either; yet both SW and LEE antigens are suppressed while MEL antigen remains as effective with adjuvant as without.

One can suggest that the injection of antigen at two-daily intervals was sufficient to bind all of the antibody being produced from the cells stimulated by the adjuvant depot. Antigen–antibody complexes formed in antibody excess are known to markedly depress the primary antibody response (Uhr and Baumann, 1961) and the same phenomenon has been observed in rabbits with influenza virus–antibody complexes formed in antibody excess (Webster, unpublished observations). Alternatively the depression may have been due to the formation of antigen–antibody complexes in antigen excess, giving rise to a hypersensitive state (Germuth and McKinnon, 1957) with no circulating antibody. These may be possible explanations of the depressed rates and levels of initial antibody formation with LEE and SW antigens but further investigation is required to explain the 'normal' response obtained with MEL antigen in adjuvant.

The increase in antibody levels on secondary stimulation is generally assumed to be an increase in the amount of antibody, but the observations indicate that the increase was both in the avidity and in the type of antibody. The instructive hypothesis in its original form has no explanation for increased titres in the secondary response. Modifications and extensions of this theory (Monod, 1959; Pappenheimer, Scharff and Uhr, 1958) postulate a permease-like induced mechanism capable of trapping antigen and thus rendering the same number of cells more effective as antibody producers on anamnestic stimulation. The selective hypothesis accounts readily for increased titres, by further proliferation of the competent clones originally stimulated by the primary antigenic dose. Heterogeneity is explained by the variable period of time spent on the template during secondary and tertiary folding of the peptide chain (Pauling, Pressman and Campbell, 1944; Karush, 1957, 1958) according to the instructive model, and by the range of cells destined to produce γ-globulin of variable complementariness to the stimulating antigen. Neither theory envisages shifts in the distribution of the antibody population, in fact, neither is elastic enough to accommodate the observed increase in avidity following secondary stimulation.

Differences in the maximal levels of antibody obtained by the routes and schedules tested, which show no sign of increased levels of antibody on further stimulation by the same route, suggest that either the total number of cells has been stimulated under these conditions or that the size of the clone is governed by the route and schedule of vaccination. The first suggestion could fit in with the instructive theory provided some genetic qualifications are incorporated into it, but in a selective scheme there seems to be no reason for one clone of cells being larger than another if the same homeostatic mechanism is supposed to limit their proliferation.

The inability to fit the collected data into the selective, the instructive, or any of the current minor theories of antibody production does not do more in itself than show up the areas where extension and qualification is needed to keep these hypotheses viable. At the same time, the indirect nature of the evidence underscores some of the weaknesses of conventional serological techniques. Whereas the theories argue in terms of number and

avidity (i.e. structure) of antibody molecules, the tests cannot do better than record some unknown combination of these two, or even worse in the case of heterogeneous populations of antibody, an average value giving no insight into the distribution of antibodies of different avidity. The immediate task, then, is to apply such methods to this collection of sera as to allow more direct and quantitative statements about the antibody response and this will be attempted in subsequent papers in this series.

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REFERENCES

ALEXANDER, J. (1931). 'Some intracellular aspects of life and disease.' *Protoplasma, Wien*, **14**, 296.

BREINL, F. and HAUROWITZ, F. (1930). 'Chemische untersuchung des präzipitates aus hämoglobin und anti-hämoglobin-serum und bemerkungen über die natur de antikörper.' *Z. physiol. Chem.*, **192**, 45.

BURNET, F. M. (1935). 'Influenza virus isolated from an Australian epidemic.' *Aust. med. J.*, **ii**, 651.

BURNET, F. M. (1957). 'A modification of Jerne's theory of antibody production using the concept of clonal selection.' *Aust. J. Sci.*, **20**, 67.

EHRLICH, P. (1900). 'On immunity with special reference to cell life.' *Proc. roy. Soc.*, B, **66**, 424.

FAZEKAS DE ST. GROTH, S. (1949). 'Modification of virus receptors by metaperiodate. I. The properties of IO_4-treated red-cells.' *Aust. J. exp. Biol. med. Sci.*, **27**, 65.

FAZEKAS DE ST. GROTH, S. (1961). 'Evaluation of quantal neutralization tests.' *Nature (Lond.)*, **191**, 891.

FAZEKAS DE ST. GROTH, S. (1962). 'The neutralization of viruses.' *Advanc. Virus Res.*, **9**, 1.

FAZEKAS DE ST. GROTH, S. and GRAHAM, D. M. (1954). 'The production of incomplete virus particles among influenza strains: experiments in eggs.' *Brit. J. exp. Path.*, **35**, 60.

FAZEKAS DE ST. GROTH, S., GRAHAM, D. M. and JACK, I. (1958). 'The serology of mumps infections. 1. A new source of antigen and a simplified complement fixation test.' *J. Lab. clin. Med.*, **51**, 883.

FAZEKAS DE ST. GROTH, S. and WEBSTER, R. G. (1961). 'Methods in immunochemistry of viruses. 1. Equilibrium filtration.' *Aust. J. exp. Biol. med. Sci.*, **39**, 549.

FAZEKAS DE ST. GROTH, S. and WEBSTER, R. G. (1963). 'Neutralization of animal viruses. IV. Parameters of the influenza virus-antibody system.' *J. Immunol.*, **90**, 151.

FAZEKAS DE ST. GROTH, S. and WEBSTER, R. G. (1964). 'The antibody response.' *Ciba Foundation Symposium on the Cellular Biology of Myxovirus Infections*, p. 246.

FAZEKAS DE ST. GROTH, S. and WHITE, D. O. (1958). 'An improved assay for the infectivity of influenza virus.' *J. Hyg. (Camb.)*, **56**, 151.

FAZEKAS DE ST. GROTH, S., WITHELL, J. and LAFFERTY, K. J. (1958). 'An improved method for neutralizing antibodies against influenza viruses.' *J. Hyg. (Camb.)*, **56**, 415.

FRANCIS, T. (1940). 'A new type of virus isolated from epidemic influenza.' *Science*, **92**, 405.

GERMUTH, F. G. and MCKINNON, G. E. (1957). 'Studies on the biological properties of antigen-antibody complexes. 1. Anaphylactic shock induced by soluble antigen-antibody complexes in unsensitized normal guinea pigs.' *Bull. Johns Hopk. Hosp.*, **101**, 13.

JERNE, N. K. (1955). 'The natural-selection theory of antibody formation.' *Proc. nat. Acad. Sci. (Wash.)*, **41**, 849.

KARUSH, F. (1957). 'The role of the disulfide bonds in antibody specificity.' *J. Amer. chem. Soc.*, **79**, 5323.

KARUSH, F. (1958). *Serological and Biochemical Comparison of Proteins* (Ed. by W. H. Cole). Rutgers University Press, New Brunswick.

LEDERBERG, J. (1959). 'Genes and antibodies.' *Science*, **129**, 1649.

MONOD, J. (1959). *Cellular and Humoral Aspects of the Hypersensitive States* (Ed. by H. S. Lawrence), p. 628. Hoeber, New York.

MUDD, S. (1932). 'A hypothetical mechanism of antibody formation.' *J. Immunol.*, **23**, 423.

NUSSENZWEIG, R. S., MERRYMAN, C. and BENACERRAF, B. (1964). 'Electrophoretic separation and properties of mouse antihapten antibodies involved in passive cutaneous anaphylaxis and passive hemolysis.' *J. exp. Med.*, **120**, 315.

PAPPENHEIMER, A. M., SCHARFF, M. and UHR, J. W. (1958). 'Delayed hypersensitivity and its possible role in antibody formation.' *Henry Ford Hospital International Symposium. Mechanisms of Hypersensitivity* (Ed. by J. H. Sharffer), p. 417. Churchill, London.

PARAF, A., FOUGERAU, M. and BUSSARD, A. (1963). In: *La Tolérance Acquise et Tolérance Naturelle à Légard de Substances Antigéniques Définies* (Ed. by A. Bussard), p. 97. CNRS, Paris.

PAULING, L. (1940). 'A theory of the structure and process of formation of antibodies.' *J. Amer. chem. Soc.*, **62**, 2643.

PAULING, L., PRESSMAN, O. and CAMPBELL, O. H. (1944). 'The serological properties of simple substances. VI. The precipitation of a mixture of two specific antisera by a dihaptenic substance containing the two corresponding haptenic groups; evidence for the framework theory of serological precipitation.' *J. Amer. chem. Soc.*, **66**, 330.

SHOPE, R. E. (1931). 'Swine influenza. 1. Experimental transmission and pathology.' *J. exp. Med.*, **54**, 349.

Svehag, S. and Mandel, B. (1964). 'The formation and properties of poliovirus neutralizing antibody. 1. 19S and 7S antibody formation: differences in kinetics and antigen dose requirement for induction.' *J. exp. Med.*, **119**, 1.

Taliaferro, H. and Taliaferro, L. G. (1961). 'Intercellular transfer of gamma-1 and gamma-2 Forssman haemolysins.' *Proc. nat. Acad. Sci.* (*Wash.*), **47**, 713.

Talmage, D. W. (1957). 'Allergy and immunology.' *Annu. Rev. Med.*, **8**, 239.

Uhr, J. W. and Baumann, J. B. (1961). 'Antibody formation. 1. The suppression of antibody formation by passively administered antibody.' *J. exp. Med.*, **113**, 935.

Uhr, J. W. and Finkelstein, M. S. (1963). 'Antibody formation. IV. Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage ØX 174.' *J. exp. Med.*, **117**, 457.