ADJUVANTS IN IMMUNIZATION WITH INFLUENZA VIRUS VACCINES

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The immunological response to a number of antigens can be improved by the addition of certain extraneous materials. For example, antitoxic immunization can be intensified by adding tapioca, calcium chloride, or bacterial vaccines (1); purified Forssman antigen which has little immunizing capacity alone is an efficient antigen when injected in combination with pig serum (2); sensitization to serum proteins is enhanced following intracutaneous injection of streptococci (3); immunization following injections of beef lens and ragweed pollen is increased when combined with staphylotoxin (4); and the response to horse serum, typhoid bacilli, diphtheria toxoid (5), single chemical compounds, and protein conjugates (6) can be heightened when injected with killed tubercle bacilli suspended in paraffin oil and a lanolin-like substance.

It was briefly reported (7) recently that the immunizing capacity of influenza virus vaccines was greatly enhanced and prolonged when the virus was injected in combination with an adsorption base and killed acid-fast bacteria suspended in paraffin oil. The present paper describes a further study of various factors that influence immunization with influenza virus suspensions in experimental animals.

Methods

Virus Suspensions.—The PR8 strain (8) of influenza A virus was used throughout these experiments. Suspensions of the virus were prepared by inoculating infected allantoic fluid in dilution of 10^{-4} into the allantoic sac of 11-day-old chick embryos. After incubation at 37°C. for 48 hours, the eggs were placed at 4°C. overnight, and blood-free allantoic fluid was removed the following morning. The fluid was cleared by a short centrifugation at 2000 R.P.M. and stored at −72°C. until used. Concentrated suspensions of the virus were obtained from the allantoic fluid by high-speed centrifugation or by adsorption of the virus onto adult chicken red blood cells and subsequent elution in buffer solution (9).

Immunity Test Following Vaccination.—Young adult mice of the Rockefeller Institute strain were used to test the immunizing capacity of the vaccines. Groups of mice were injected subcutaneously (0.5 cc.) with the vaccine to be tested together with comparable groups which received control materials. At various intervals thereafter the mice were tested for resistance to intranasal instillation of graded amounts of PR8 allantoic fluid. The lungs of the mice were examined after death for the presence and extent of consolidation or on the 10th day following inoculation, at which time all of the surviving mice were killed. The degree of consolidation was expressed in terms of the relative volume of the total lung consolidated as described by

Horsfall (10): 5 = complete consolidation with death, 4 = complete consolidation with survival, 3, 2, 1 = relative proportion of the total lung consolidated.

Tests for Circulating Antibodies.—The capacity of the vaccines to elicit antibodies was tested in ferrets and in rabbits. The animals were bled from the heart under light ether anesthesia before inoculation and at frequent intervals thereafter. The blood was allowed to clot at room temperature for 1 hour and left overnight at 4°C. Serum was then separated and stored at 4°C.

The antibody levels of the sera were determined by the agglutination inhibition test (11), and in some instances neutralization (10) and complement fixation tests (12) were also done. Each of these tests has been fully described. Serial serum dilutions in all of the tests were made by delivering an accurately measured volume of serum to the diluent and mixing with a fresh pipette, as ordinarily done in infectivity tests with virus suspensions. This method of diluting was essential even in the *in vitro* tests because of the high titers of many of the sera. Furthermore, a higher degree of accuracy in the titrations was thereby attained as evidenced by the reproducibility of the titers in repeat tests. A standard ferret serum as well as rabbit sera from preceding bleedings was included in each test to correct for differences in titer which occur in tests done at different times. Usually several groups of serial bleedings were tested at the same time.

EXPERIMENTAL

Effect of Paraffin Oil and Killed Tubercle Bacilli on Immunizing Capacity of Influenza Virus in Mice.—In a first experiment the immunizing capacity of a single subcutaneous injection of PR8 allantoic fluid in combination with killed tubercle bacilli suspended in paraffin oil and an absorption base was tested in mice.

An allantoic fluid suspension of PR8 virus (agglutination titer 1:388), which had been rendered non-infectious for mice by the addition of formalin (0.1 per cent final concentration), was blended with paraffin oil containing killed tubercle bacilli and an adsorption base known as Falba.[1] The tubercle bacilli, which were of the virulent human Jamaica No. 22 strain, were kindly supplied by Dr. M. W. Chase. They were heated at 100°C. in the Arnold sterilizer for 30 minutes and then incorporated in sterile paraffin oil. The Falba was melted and blended by means of an automatic stirrer (13) with the paraffin oil (Seybold viscosity 175 to 180 seconds at 100°F. (14)) containing tubercle bacilli. The allantoic fluid was then added dropwise with continuous stirring. Each cubic centimeter of the emulsion contained 0.4 cc. of the allantoic fluid, 0.4 cc. of paraffin oil, 0.2 cc. of Falba, and 1.4 mg. of tubercle bacilli. The final emulsion had a thick, creamy consistency and remained stable after standing at 4°C. for at least 6 months.

The virus-adjuvant emulsion was injected subcutaneously (0.5 cc.) into a group of mice. For controls a comparable group of mice received the same amount of the PR8 allantoic fluid in saline, and a third group received only saline. The three groups of mice were tested for resistance to intranasal instillation of graded amounts of PR8 allantoic fluid at 4, 8, 16, and 26 weeks after vaccination as described under Methods. The same PR8 allantoic fluid which had been kept frozen was used in each test.

Four weeks after vaccination (Fig. 1), the mice that received the virus in saline were resistant to roughly 100 M.I.D. (minimal infectious doses) of virus, whereas the mice inoculated with the virus-adjuvant mixture were resistant to

[1] *Falba* is said to be a mixture of beeswax, paraffin oils of varying viscosities, and oxycholesterins extracted from lanolin. (Distributed by Pfaltz & Bauer, Inc., New York.)

about 1,000,000 M.I.D. of virus. The immunity elicited by the virus in saline was less at 8 and 16 weeks, and at 26 weeks there was little difference between the lung lesions of the control and vaccinated mice. The immunity called forth by the virus-adjuvant emulsion, on the other hand, had remained at a high level at 8 weeks, and even after 26 weeks, though less than at 4 or 8 weeks, it was still somewhat greater than the immunity elicited by the virus in saline at 4 weeks. Manifestly these adjuvants not only greatly increased the immunizing capacity of the virus but maintained the immunity at a high level over a long period of time.

The virus-adjuvant emulsion regularly produced a firm subcutaneous nodule at the site of inoculation. The nodules varied in size from ½ to 1½ cm. in diameter and persisted for several months, thereafter gradually becoming smaller and finally disappearing. Although the skin overlaying the nodule was frequently inflamed, ulceration rarely occurred. On cross-section the nodules consisted of a cyst containing the creamy, oily material of the original inoculum. A thick wall of reactive tissue containing large mononuclear cells, lymphocytes, and polymorphonuclear leukocytes surrounded the nodules.[2]

Antibody Response to Virus-Adjuvant Emulsions in Rabbits.—PR8 allantoic fluid and the adjuvants used in the preceding experiment were now tested for capacity to induce circulating antibodies in rabbits. In addition a portion of the same PR8 allantoic fluid was concentrated by means of red cell absorption and elution and this virus preparation was concurrently tested with and without the adjuvants.

One hundred and fifty cc. of PR8 allantoic fluid was mixed with 1.5 cc. of adult chicken red blood cells which had been washed three times and packed by light centrifugation. The allantoic fluid and red cells were kept at 4°C. for 30 minutes. The red cells were then removed by light centrifugation and resuspended in 15 cc. of M/50 phosphate buffer solution pH 6.9. The mixture was left at 37°C. for 3 hours with frequent gentle shaking. The red cells were then discarded following light centrifugation. Formalin was added (0.1 per cent final concentration) and the virus preparation was left at 4°C. overnight. Agglutination tests showed that the original allantoic fluid had a titer of 1:388 whereas the titer of the concentrated virus preparation was 1:2200.

The concentrated virus suspension thus obtained and the untreated PR8 allantoic fluid were blended with paraffin oil, tubercle bacilli, and Falba as described in the preceding experiment. For control a portion of the virus suspension was mixed with saline instead of the adjuvants. The mixtures were then inoculated subcutaneously (1 cc.) into groups of 3 adult domestic rabbits. The upper part of the back was used as the inoculation site. Nodules similar to those already described in mice developed at the site of inoculation of the virus-adjuvant emulsions. The rabbits were bled before inoculation and at frequent intervals thereafter. The titers of circulating antibodies were determined by means of agglutination inhibition and complement fixation tests.

[2] It seemed possible that the macrophages surrounding the inoculum might be an important factor in the increased antibody response. Consequently, several materials (aleuronat, broth, plain diphtheria toxin, and typhoid bacilli) which are known to stimulate the production of mononuclear cells (15) were tested in combination with PR8 influenza virus. Little or no enhanced immunity or antibody response to the virus was obtained.

The antibody titers called forth by the untreated allantoic fluid virus vaccines are shown in Fig. 2. The titers of the individual rabbits as well as the geometric mean of each group (heavy lines) are graphically presented. The titers of the rabbits inoculated with the virus-saline suspension were highest 2

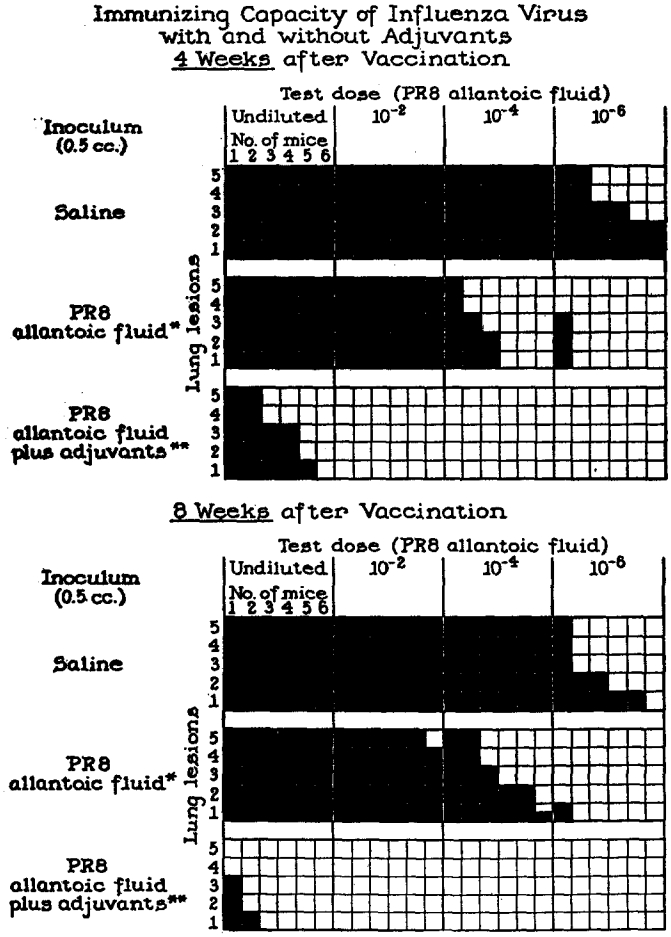


FIG. 1

weeks after inoculation, followed by a progressive fall to low levels within 16 weeks. The virus-adjuvant emulsion, on the other hand, elicited the highest titers of antibodies 4 to 6 weeks after inoculation and they were at least 10 times higher than those elicited by the virus-saline control. Furthermore, the titers remained at a high level up to 26 weeks after vaccination (the longest period tested). Indeed, the levels of circulating antibodies in 2 of the rabbits

actually increased after 16 weeks. It will be noted that the titer of each rabbit of the latter group after 26 weeks was better than the highest titer elicited by

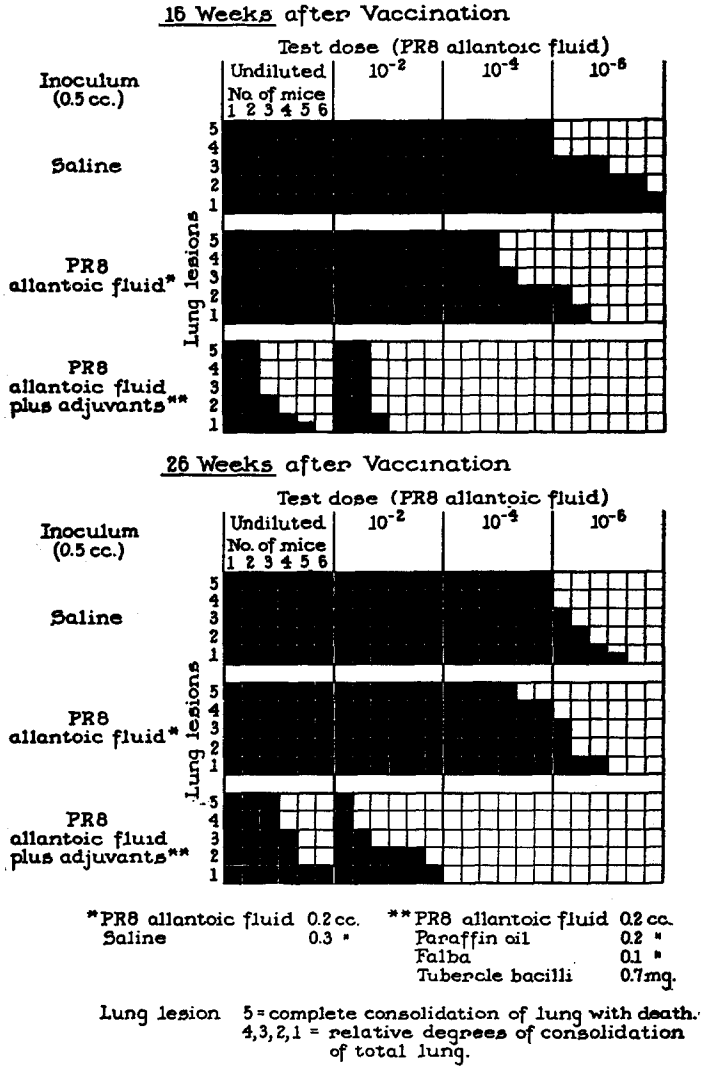
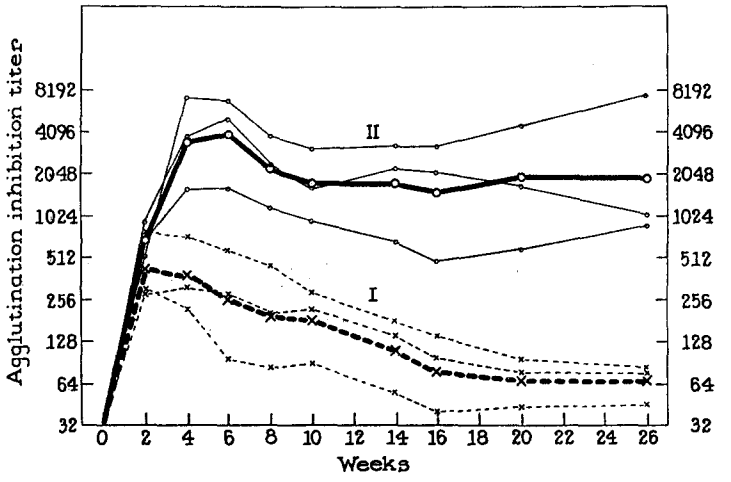


FIG. 1—*continued*

the virus in saline (2 weeks). Complement fixation tests with these sera gave similar results and they need not be presented in detail.

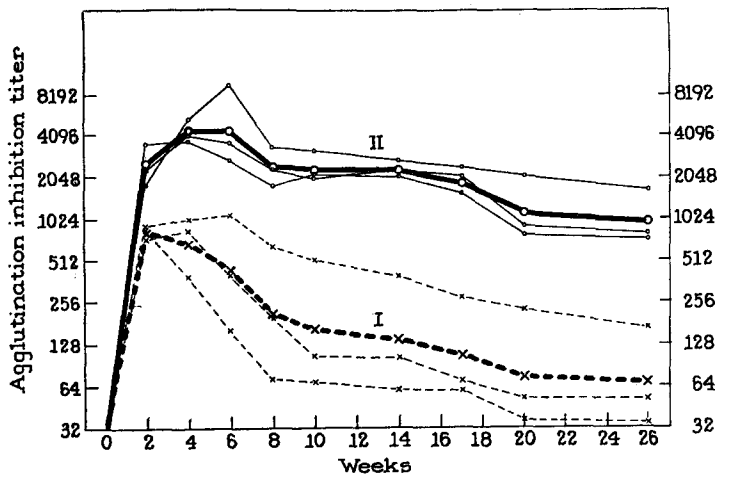
The results obtained with the virus preparation concentrated by red cell adsorption and elution are shown in Fig 3. It will be seen that the antibody



I. PR8 allantoic fluid 0.4 cc.
Saline 0.6 "

II. PR8 allantoic fluid 0.4 cc.
Paraffin oil 0.4 "
Falba 0.2 "
Tubercle bacilli 1.4 mg.

FIG. 2. Serum antibody response in rabbits following inoculation of PR8 allantoic fluid with and without adjuvants. The heavy solid and heavy broken lines represent the geometric mean titers of each group of rabbits.



I. PR8 virus (concentrated) 0.4 cc.
Saline 0.6 "

II. PR8 virus (concentrated) 0.4 cc.
Paraffin oil 0.4 "
Falba 0.2 "
Tubercle bacilli 1.4 mg.

FIG. 3. Serum antibody response in rabbits following inoculation of PR8 virus material obtained from allantoic fluid by red cell adsorption and elution (see text) and injected with and without adjuvants.

response to the virus suspension was likewise greatly enhanced and maintained at a high level by the adjuvants, although the titers attained were no higher than when the untreated allantoic fluid was used as the source of virus (Fig. 2). The concentrated virus without the adjuvants induced slightly higher titers of antibodies than the untreated allantoic fluid within 2 weeks after inoculation, but the titers dropped rapidly after 2 weeks and within 8 weeks they were not significantly different from the titers elicited by the untreated allantoic fluid.

Antibody Response in Ferrets.—The antibody response in ferrets following intranasal inoculation of infective PR8 virus was compared with the amount

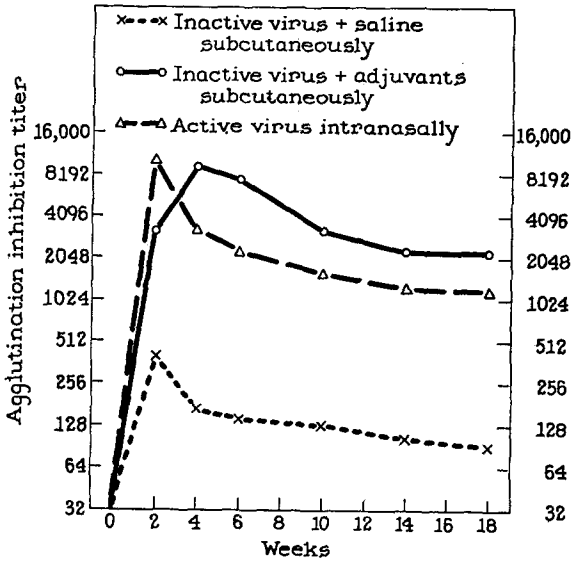


FIG. 4. Comparison of mean serum antibody titers in ferrets following infection with PR8 virus and after subcutaneous inoculation of formalinized PR8 virus with and without adjuvants. Each curve represents the mean response of a group of four ferrets.

of antibodies elicited by a single subcutaneous inoculation of formalinized PR8 virus with and without adjuvants in the next experiment.

PR8 allantoic fluid was diluted to $10^{-5.0}$ and instilled intranasally (0.5 cc.) into each of 4 normal ferrets. The allantoic fluid had an infectivity titer of $10^{-6.2}$ when tested in mice. A portion of the same allantoic fluid was formalinized (0.1 per cent) and then blended with paraffin oil, tubercle bacilli, and Falba as described in the preceding experiments. For control the formalinized allantoic fluid was mixed with saline instead of the adjuvants. The virus-adjuvant emulsion and the virus-saline suspension were each inoculated subcutaneously (2 cc.) into groups of 4 ferrets. All ferrets were bled before inoculation and at various intervals thereafter. The serum antibody titers were determined by means of the agglutination inhibition test.

The serum titers of the individual ferrets in each group were quite similar and hence only the mean titer of each group is shown in Fig. 4. As observed

in rabbits the formalinized virus in saline elicited the highest antibody levels within 2 weeks after inoculation followed by a rapid decrease within 4 weeks and then a more gradual decrease up to 18 weeks (the longest period tested). The formalinized virus with the aid of the adjuvants induced much more circulating antibody than the virus in saline and the titers remained at a high level for the duration of the experiment. The highest titers were elicited 4 weeks after inoculation and at this time they were about 50 times higher than the titers produced by the virus in saline. Infection of the ferrets with active influenza virus also produced high titers of antibodies (Fig. 4, broken line). The highest titers were observed within 2 weeks after inoculation followed by a decrease to levels which were slightly lower than those elicited by subcutaneous inoculation of the formalinized virus with the adjuvants.

Mycobacterium butyricum and Paraffin Oil as Adjuvants.—A saprophytic acid-fast organism, *Mycobacterium butyricum*, was now substituted for the tubercle bacilli in emulsions of PR8 allantoic fluid, Falba, and paraffin oil.

A culture of *Mycobacterium butyricum*, No. 362, was obtained from the American Type Collection. A profuse growth of the bacteria was obtained in broth pH 7.4 after incubation at 37°C. for 3 days. The bacteria were removed by centrifugation, washed 3 times in distilled water, and then inactivated by heating at 100°C. for 30 minutes in the Arnold sterilizer. They were then frozen, dried, and kept in sealed ampoules until used.

PR8 allantoic fluid, which had been inactivated with formalin, was combined with the heat-killed *Myco. butyricum* suspended in paraffin oil and Falba in the same proportions described in the preceding experiments. The allantoic fluid had an agglutination titer of 1:338. A comparable emulsion containing the allantoic fluid, paraffin oil, and Falba without the butter bacilli was also prepared. For control the allantoic fluid was mixed with physiological saline instead of the adjuvants. One cc. of each preparation was then injected subcutaneously into groups of 4 normal domestic rabbits. The rabbits were bled before inoculation and at various intervals thereafter. The serum antibody levels of each rabbit were determined by means of the agglutination inhibition test.

The mean antibody titers of each group of rabbits are shown in Fig. 5. The PR8 allantoic fluid in saline induced levels of antibody comparable to those previously obtained (see Fig. 2). The antibody titers of the rabbits that received the virus combined with the butter bacilli, paraffin oil, and Falba continued to rise sharply up to 12 weeks after inoculation. At this time the titers were about one hundredfold higher than the titers induced by the virus in saline. Furthermore, the titers remained at this level up to 24 weeks (the longest period of test). The virus combined with paraffin oil and Falba was much less effective in producing antibodies although the titers remained at a constant level up to 24 weeks.

A comparison of the results obtained with the *Myco. butyricum* and with the tubercle bacilli (Figs. 2 and 5) indicates that the former is considerably more effective in enhancing the antibody response to the virus. Freund and Walter (16) recently reported that killed timothy bacilli promoted antibody formation

and sensitization to horse serum almost as effectively as tubercle bacilli. The emulsion containing the butter bacilli produced firm nodules at the site of inoculation which were similar to those evoked by the tubercle bacilli emulsions. The nodules persisted up to about 10 weeks after inoculation and then gradually became softer and smaller. The emulsion without the butter bacilli produced much smaller nodules and they were not palpable 4 weeks after inoculation.

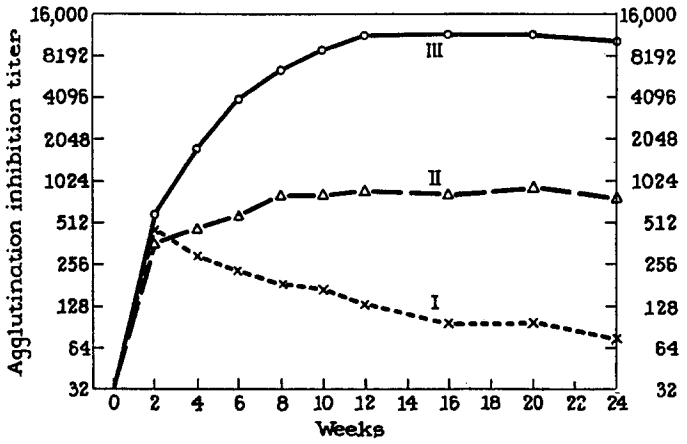


FIG. 5. Mean antibody titers in rabbits induced by PR8 allantoic fluid in combination with *Myco. butyricum*, paraffin oil, and Falba. Each curve represents the mean response of a group of four rabbits.

I.	PR8 allantoic fluid	0.4 cc.
	Saline	0.6 cc.
II.	PR8 allantoic fluid	0.4 cc.
	Paraffin oil	0.4 cc.
	Falba	0.2 cc.
III.	PR8 allantoic fluid	0.4 cc.
	Paraffin oil	0.4 cc.
	Falba	0.2 cc.
	Myco. butyricum	1.4 mg.

Table I shows the results of comparative agglutination inhibition, complement fixation, and neutralization tests with the rabbit sera obtained 12 weeks after vaccination with the virus in saline and with the virus combined with the butter bacilli in paraffin oil and Falba (see Fig. 5). In the mouse neutralization test 31 M.L.D. of virus (PR8 allantoic fluid) was mixed with serial dilutions of serum in steps of $10^{0.5}$ and the mixtures left at 4°C. overnight (17). Each dilution was then instilled intranasally (0.05 cc.) into 6 mice and the serum dilution end point determined by the 50 per cent mortality method (18). The mean antibody titers of the two groups of rabbits showed about a

hundredfold difference when tested by the agglutination inhibition and complement fixation tests and about a five hundredfold difference by the neutralization test.

Complement fixation tests with sera from rabbits inoculated with allantoic fluid suspensions of virus and adjuvants revealed antibodies directed not only against the virus but also against antigenic material present in normal allantoic fluid.

The sera obtained from the rabbits inoculated with PR8 allantoic fluid and with the concentrated PR8 virus suspension prepared by red cell adsorption and elution in the experiments already described (see Figs. 2 and 5) were tested for antibodies against PR8 allantoic fluid and

TABLE I

Comparative Agglutination Inhibition, Complement Fixation, and Neutralization Tests with Rabbit Sera

Inoculum (1 cc.)	Rabbit No.	Serum antibody titer*		
		Agglutination inhibition test	Complement fixation test	Neutralization test
PR8 allantoic fluid	a	64	3	178
Saline	b	315	37	1800
	c	138	21	1000
Mean		**138**	**13**	**670**
PR8 allantoic fluid	d	10,800	1910	398,000
Paraffin oil	e	7,200	958	180,000
Falba	f	12,400	2048	560,000
Myco. butyricum				
Mean		**10,100**	**1560**	**340,000**

* The sera were obtained 12 weeks after inoculation. Titers expressed as the reciprocal of the serum dilution end point.

normal allantoic fluid by means of the complement fixation test. The marked procomplementary effect of allantoic fluid[3] previously described (12) necessitated separate titrations of the complement in the presence of PR8 allantoic fluid, normal allantoic fluid, and saline. The 50 per cent hemolytic end point was determined in each titration by means of a photoelectric densitometer (12) and $2\frac{1}{4}$ fifty per cent units of complement were used in the test.

The results are summarized in Table II. The PR8 allantoic fluid in saline induced antibodies only against the PR8 allantoic fluid and none could be de-

[3] Enhancement of the hemolytic activity of complement has been also observed with other chick embryo materials including amniotic fluid and saline extracts of the whole embryo or yolk sac. Considerable variation in the complement unit is often encountered in titrations in the presence of chick embryo fluids or extracts from different sources and hence separate titrations of the complement in the presence of each antigen and control material are required for an accurate appraisal of the complement unit.

tected when normal allantoic fluid was used as the antigen. With the aid of the adjuvants, however, much higher titers of antibodies against the PR8 allantoic fluid were elicited and fixation was now obtained with normal allantoic fluid as well. The latter titers were considerably lower, however, than those obtained with the PR8 allantoic fluid antigen. No fixation was observed in the saline controls. The concentrated virus suspension in saline elicited low titers of

TABLE II

Complement Fixation Tests with Sera from Rabbits Inoculated with Influenza Virus and Adjuvants

Inoculum			Serum antibody titer								
			4 wks.			8 wks.			16 wks.		
			Complement-fixing antigen*								
Source of virus	Material added	Rabbit No.	PR8 allantoic fluid	Normal allantoic fluid	Saline	PR8 allantoic fluid	Normal allantoic fluid	Saline	PR8 allantoic fluid	Normal allantoic fluid	Saline
PR8 allantoic fluid	Saline	a	32	—	—	12	—	—	10	—	—
		b	158	—	—	79	—	—	37	—	—
		c	60	—	—	34	—	—	20	—	—
	Paraffin oil	d	182	42	—	1175	158	—	1670	182	—
	Falba	e	588	39	—	1270	120	—	1350	91	—
	Myco. butyricum	f	779	84	—	2520	338	—	2700	388	—
Concentrated PR8 virus (Red cell adsorption and elution)	Saline	g	49	—	—	23	—	—	16	5	—
		h	120	6	—	104	5	—	52	7	—
		i	60	—	—	30	—	—	11	6	—
	Paraffin oil	j	315	12	—	223	13	—	194	7	—
	Falba	k	512	28	—	779	39	—	388	24	—
	Myco. tuberculosis	l	388	15	—	223	12	—	147	7	—

* PR8 and normal allantoic fluids diluted 1:5 with saline.

Complement, 2¼ fifty per cent hemolytic units as determined by titration in the presence of each antigen and saline.

— = titer less than 1:4.

antibodies against normal allantoic fluid and these were not detected in 2 of the rabbits until 16 weeks after inoculation. The concentrated virus in the presence of the adjuvants induced higher titers of antibodies against both PR8 and normal allantoic fluids.

Sesame Oil and Myco. butyricum as Adjuvants.—The striking effect of the butter bacilli in the emulsions used in the preceding experiments suggested the possibility that suspensions of influenza virus and these bacilli in a vegetable oil might enhance the response to the virus. This was tested in the following experiment.

Formalinized PR8 allantoic fluid was spun at 20,000 R.P.M. for 1 hour in an air-driven centrifuge of the vacuum type (19). The supernatant fluid was discarded and the pellets of sediment resuspended in the original volume of sesame oil. Dried heat-killed butter bacilli were then added to the mixture so that each cubic centimeter contained 1 mg. A comparable mixture was also prepared containing typhoid bacilli instead of the butter bacilli. Typhoid, Para A, and Para B bacilli were obtained by centrifuging vaccine procured from the Department of Health, City of New York, and resuspending the pellets in sesame oil containing the PR8 virus. Each cubic centimeter of the mixture contained the bacteria sedimented from 1 cc. of vaccine. For controls the same amount of virus sediment was resuspended in sesame oil and in buffer solution. One cc. of each mixture was then inoculated subcutaneously into groups of 5 normal, domestic rabbits. Ten weeks after inoculation each rabbit received a

TABLE III

Effect of Sesame Oil, Myco. butyricum, and Typhoid Bacilli on the Serum Antibodies and Immunity Elicited by Sedimented PR8 Virus

Inoculum (1 cc.)	Mean serum antibody titer (rabbits)*									Immunity test (mice)
	1st inoculation			Weeks			2nd inoculation ↑			6 wks. after inoculation
	0	2	4	6	8	10	12	14	18	M.L.D. resistance
Virus‡ Buffer solution	<32	158	128	79	60	60	239	104	69	15
Virus Sesame oil	<32	69	97	97	79	64	275	208	112	25
Virus Sesame oil Typhoid bacilli	<32	128	138	147	120	91	388	223	138	100
Virus Sesame oil *Myco. butyricum*	<32	588	479	362	256	315	1100	722	362	500

* Five rabbits per group.

‡ Virus sediment from PR8 allantoic fluid after centrifugation at 20,000 R.P.M. for 1 hour.

second injection of the same mixture which had been freshly prepared. The rabbits were bled before inoculation and at intervals of 2 weeks thereafter. The serum antibody titers were determined by means of the agglutination inhibition test. In addition 0.5 cc. of each mixture was inoculated subcutaneously into mice. Six weeks after inoculation, the control uninoculated mice and the vaccinated mice received active PR8 allantoic fluid intranasally in serial tenfold dilutions from 10^{-1} to 10^{-5}. Eight mice were used for each dilution. The approximate M.L.D. resistance of each group was determined by comparing the 50 per cent mortality end points in the control and vaccinated mice.

The mice and rabbits that received the virus–sesame oil–*Myco. butyricum* mixture developed firm nodules at the site of injection. They varied in size from ½ to 3 cm. in diameter and persisted for about 2 months although gradually becoming smaller and finally disappearing. No nodules were produced by the other mixtures.

Table III summarizes the results of the serum titrations. The mean serum antibody titers in rabbits that received the virus suspended in sesame oil were somewhat lower 2 weeks after the first inoculation and slightly higher after the second inoculation than the titers of the rabbits that received the virus suspended in buffer solution. The addition of typhoid bacilli increased the antibody titers slightly and this was also reflected in an increased immunity in mice. The best response, both in rabbits and mice, was obtained with the mixtures containing the butter bacilli. The antibody titers, however, were much lower than those attained when the emulsions of butter bacilli and paraffin oil were used as adjuvants (see Fig. 5). Furthermore, the antibody titers gradually decreased after the peak at 2 weeks after inoculation and were not maintained at a constant level as already observed with the paraffin oil emulsions. It will be noted that 2 injections of the virus in buffer solution failed to induce as much antibody as a single injection of the virus and butter bacilli suspended in sesame oil.

DISCUSSION

The mechanism of the enhanced response to a virus antigen when combined with the adjuvants herein described is obviously complex. Under the ordinary conditions of inoculation with watery suspensions of antigen the antigenic stimulus is apparently short-lived, for the antigen is absorbed by the body and probably destroyed within a short period of time. This is reflected in the characteristic antibody response in which the highest titers are reached within 2 weeks after inoculation and are followed by a progressive decrease to low antibody levels thereafter. The antibody response to inoculation of the antigen in the form of an emulsion containing acid-fast bacteria and paraffin oil, on the other hand, continues to increase up to 1 to 3 months after inoculation, reaching extraordinarily high titers. More significant, however, is the fact that the titers are maintained at high levels for at least 6 months.

The function of the paraffin oil and acid-fast bacteria is probably to set up a reactive tissue wall about the inoculum and thus localize and maintain the antigenic material at the inoculation site. The adsorption base (Falba) undoubtedly contributes to the tissue reaction as well as combining the watery and the oily components into a stable water-in-oil emulsion. Manifestly these conditions provide excellent opportunity for hyperimmunization by a slow continuous absorption of the antigen from the mass. It is also probable that the mononuclear cells called forth by these materials are involved in the increased production of antibodies as previously suggested (20).

Unfortunately, it does not seem likely that the materials thus far found to be effective as adjuvants can be safely used in human immunization. Induced sensitivity to tubercle bacilli is well known and the paraffin oil used in these experiments produced marked connective tissue irritation. The saprophytic

acid-fast bacilli, *Myco. butyricum*, were found to be even more effective than the tubercle bacilli as adjuvants. These organisms have the further advantages that they can be readily grown on ordinary culture media, and they have been reported to be antigenically unrelated to the tubercle bacilli (21). However, Freund and Walter (16) recently demonstrated cross-sensitivity in guinea pigs between another saprophytic bacterium, the timothy bacillus and the tubercle bacillus. Another objection to the use of saprophytic acid-fast bacilli in an oily suspension is the possible formation of miliary lesions in the lungs (22). Manifestly further investigation is required to find materials that can be safely used in human beings to promote the immunological response to subcutaneous injection of antigens.

SUMMARY

Subcutaneous inoculation of PR8 allantoic fluid, or watery suspensions of the virus obtained from allantoic fluid by high-speed centrifugation or by elution after adsorption on red cells induced serum antibodies in experimental animals, which reached the highest levels within 2 weeks after inoculation and were gradually lost thereafter. The addition of killed acid-fast bacteria (*Myco. tuberculosis* or *butyricum*), paraffin oil, and a proprietary adsorption base (Falba) to form a stable water-in-oil emulsion of influenza virus suspensions greatly enhanced and maintained immunity and antibody response to the virus. These adjuvants provided a much more effective method of increasing antibody production to the virus than the use of concentrated preparations of virus alone.

Paraffin oil and Falba without the acid-fast bacilli were less effective as adjuvants, although the antibody levels induced were higher than those produced by watery suspensions of the virus and were maintained at a constant level for at least 6 months. *Myco. butyricum* appeared to be more effective in producing antibodies against the virus than the tubercle bacilli in the emulsions of paraffin oil and Falba. Immunization with these adjuvants and suspensions of influenza virus obtained from allantoic fluid induced antibodies not only against the virus but against antigenic material contained in normal allantoic fluid, although the latter titers were considerably lower.

A suspension of influenza virus (sedimented by high-speed centrifugation) and *Myco. butyricum* in sesame oil induced about four times as much antibody as when the virus was suspended in saline, in sesame oil alone, or in combination with typhoid bacilli.

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