TABLE III. Effect of an Intrauterine Device (IUD) on the Estrous Cycle Length of Intact and Unilaterally Ovariectomized Guinea Pigs (Exp 3).

Item	Both ovaries intact			One ovary removed	
	No IUD (sham-operated)	IUD in only one horn	IUD in both horns	IUD in horn adjacent to retained ovary	IUD in horn opposite to retained ovary
No. guinea pigs	7	7	7	10	9
Operative* cycle length (days)	16.1	15.5	14.3	13.0	16.1
Subsequent* cycle length (days)	16.2	16.6	14.6	14.8	17.2
Both operative and subsequent cycle length (days)	16.2†	16.1†	14.4‡	13.9‡	16.7†

* Mean squares for the 5 treatment groups, 25.10 ($P < 0.01$); operative cycle *vs* subsequent cycle, 18.00 ($P < 0.01$); interaction, 1.92; error for treatment, 3.46; error for cycle and for interaction, 2.41.
†, ‡ Means bearing different reference marks are significantly different from each other ($P < 0.05$).

present at the time of laparotomy during the subsequent cycle (avg no., 2.9) resulted from ovulations which occurred in the presence of an IUD. There was no significant difference in CL numbers among the 5 treatment groups or between the operative and subsequent cycles, nor was there any interaction between treatment and cycle. Removal of one ovary (Groups 4, 5) during the operative cycle did not change significantly the number of ovulations per animal during the subsequent cycle (3.0 *vs* 2.7, respectively).

Summary. The effects of an intrauterine device (IUD) on the corpora lutea, estrous cycle length and ovulation rate were studied in guinea pigs. Insertion of an IUD on day 2 of an estrous cycle resulted in smaller corpora lutea in the ovary adjacent to the IUD than in the opposite ovary. The average estrous cycle length was shortened in ovarian-intact guinea pigs with an IUD in each uterine horn and in unilaterally ovariectomized guinea pigs with the IUD adjacent to the retained ovary. Estrous cycles were not shortened in ovarian-intact guinea pigs with an IUD in only one horn or in unilaterally ovariectomized guinea pigs with the IUD opposite to the retained ovary. No evidence was obtained that the IUD influenced the rate of ovulation.

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New Metabolizable Immunologic Adjuvant for Human Use. 4. Development of Highly Purified Influenza Virus Vaccine in Adjuvant 65. (31601)

A. F. WOODHOUR, D. P. METZGAR, G. P. LAMPSON, R. A. MACHLOWITZ, A. A. TYTELL, AND M. R. HILLEMAN
Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pa.

Previous reports(1-4) from this laboratory described the development and clinical evaluation of a new immunologic adjuvant, called adjuvant 65, which was applied to influenza virus vaccine. The adjuvant vaccine consisted of a water-in-oil emulsion of the aqueous vaccine in highly refined peanut oil using Arlacel A (mannide monooleate) as emulsifier and aluminum monostearate as stabilizer. A detailed analytic review of adjuvants and adjuvant activity has been recorded(5) elsewhere. Further studies of influenza-adjuvant vaccine led to the development of a highly purified aqueous influenza virus vaccine which

TABLE I. Bivalent Vaccines Tested in Animals.

Lot No.	Kind	Composition: Virus strain	CCA units per 0.5 ml dose	CCA units/mg protein* (aqueous): Individual	Combined
140-C4108	Purified—Adjuvant 65	A2/Japan/170/62	125	9135	9298
		B/Maryland/1/59	125	9460	
		Total	250		
156-C4550	Purified—Aqueous	A2/Japan/170/62	250	9135	6458
		B/Maryland/1/59	250	3780	
		Total	500		
143-C4110	Sharples—Aqueous	A2/Japan/170/62	250	N.D.	2439
		B/Maryland/1/59	250	N.D.	
		Total	500		

* Nondialyzable Lowry protein.

has been incorporated into adjuvant 65 with excellent results.

The present report describes the development of the purified influenza-adjuvant 65 vaccine and presents data on the long-term holding of adjuvant 65 influenza vaccines with respect to potency and with respect to failure to attain toxic properties. A subsequent paper (6) describes the clinical findings in studies of the purified influenza-adjuvant vaccine.

Materials and methods. Purified aqueous vaccine. The influenza viruses noted in the text were propagated by ordinary procedure in the allantoic cavity of embryonated hens' eggs. The Sharples aqueous vaccine was prepared by the ordinary Sharples concentration method. The vaccine designated as purified was subjected to both chemical and physical manipulations to achieve a high degree of purity. Inactivation of the virus was accomplished with formalin and virus standardization was by the refined hemagglutination test procedure described by Miller(7). *Adjuvant 65.* The purified vaccine was emulsified in adjuvant 65 according to procedures described earlier(1). *Potency assays.* Groups of 10 guinea pigs weighing 400-450 g were inoculated intramuscularly on 2 occasions one month apart with 0.5 ml of aqueous or adjuvant 65 vaccine. The animals were bled immediately prior to vaccination and 1 month and 2 months later. Quantitative assays for hemagglutination-inhibiting (HI) antibody were carried out on the individual sera by previously recorded methods(1,3). *Toxicity tests.* The adjuvant preparations were tested for short-term toxictiy by dermal assays in guinea pigs and by growth suppression tests in mice as previously recorded(2). *Protein determinations* were performed according to Lowry *et al*(8).

Results. Vaccines used. Three lots of vaccine were prepared as shown in Table I. Each vaccine was bivalent and contained A2/Japan/170/62 and B/Maryland/1/59 viral antigens in the chicken cell agglutination (CCA) units shown. The same purified influenza A2 vaccine lots were used to prepare the aqueous and adjuvant vaccines but the B/Md/1/59 preparations were different. The purity of the Sharples vaccine was 2439 CCA units per mg of total protein while the purified virus materials were 2.7 to 3.8 times as pure (6458 to 9298 CCA units/mg protein). The aqueous vaccines contained twice as much influenza antigen per dose as did the adjuvant vaccine.

Potency assays in guinea pigs. The geometric mean HI titers obtained in the guinea pigs after 1 or 2 doses of vaccine are shown graphically in Fig. 1. It is seen in the Figure that the purified and Sharples vaccines were roughly equal in performance in guinea pigs and that the response to the adjuvant 65 vaccine was markedly greater even though only half as much influenza antigen was used. The enhancement in serologic response by adjuvant was most apparent following the first dose of vaccine.

Antigenic stability of adjuvant 65 vaccine.

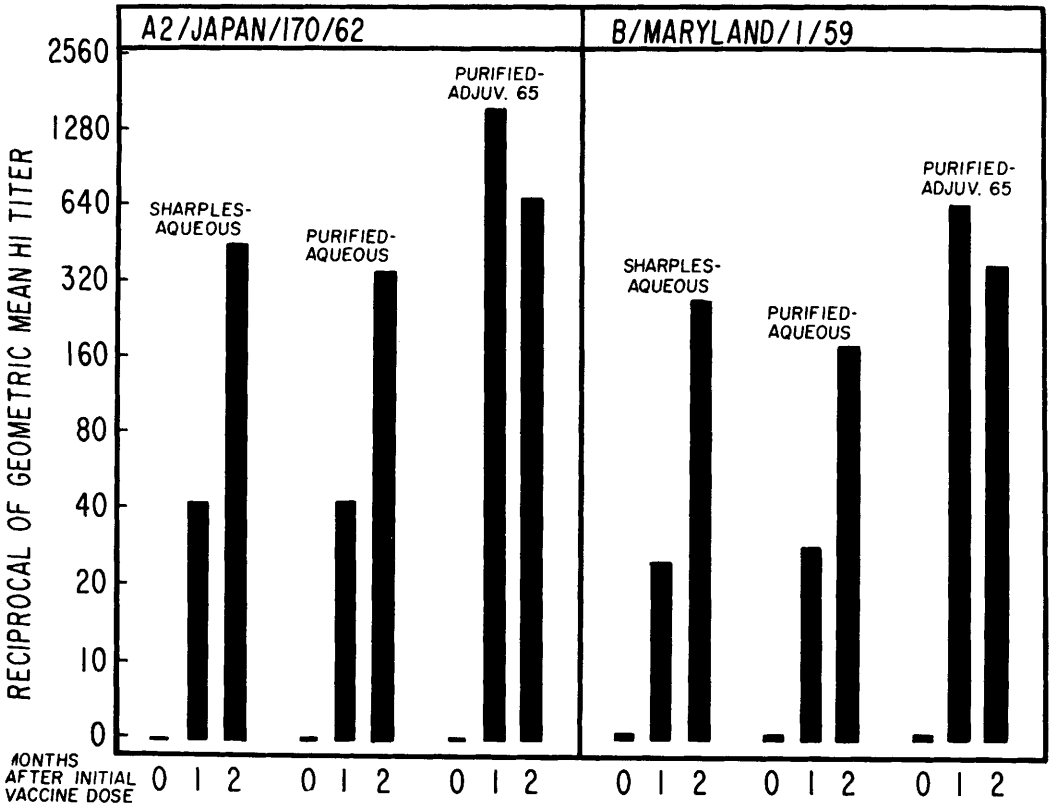

FIG. 1. Homologous antibody responses in guinea pigs to aqueous and adjuvant vaccines tested 7 months after preparation.

Table II summarizes the findings in tests for stability of immunizing potency of influenza-adjuvant 65 and the corresponding aqueous vaccines. Preparation of the vaccines was described elsewhere(1-3) and in the present report. The vaccines were tested initially within 1 month after preparation and again 7 to 41 months later. The excellent adjuvant potentiation of the vaccines was clearly demonstrated and there was no detectable loss of potency of either the aqueous or purified vaccines when stored at 4°C for the time periods shown.

Tests for development of toxicity on storage. The dermal toxicity test in guinea pigs is based on observation for development of local pathology for 14 days following inoculation intradermally in 0.1 ml amount in multiple sites in guinea pigs. Pathology is expressed in terms of skin-fold thickness (induration) in 64ths of an inch at the injection site measured at various time intervals for 14 days following injection and presence or absence of tissue necrosis. The average values found for 6 injection sites are used. Reference vaccines which have been found satisfactory in tests in man are always included for control purpose. The findings for 6 vaccine lots tested less than one month after preparation and again 7 to 41 months later are as shown in Table III. The values obtained with satisfactory control materials approximated those of the test materials and are not included in the Table. The vaccines caused a minor induration at the local site which reached a maximum around the seventh day after injection and regressed to normal or near normal by the fourteenth day. Necrosis and ulceration, which have been found by us to be caused by influenza virus vaccines in incomplete mineral oil adjuvant of acceptable quality, were not encountered. It is seen in the Table that the induration induced by the fresh compared with the corresponding

TABLE II. Antigenic Stability of Adjuvant 65 Compared with Aqueous Influenza Virus Vaccines on Long-Term Storage at 4°C. Homologous antibody responses as measured in guinea pigs.

Vaccine*				Geometric mean HI antibody titer against homologous virus							
Lot No.	Kind	Total CCA† per 0.5 ml	Age of vaccine at test (mo)	A Swine '29	A PR8 '34	A1 AA/1 '57	A2 Jap/305 '57	A2 Jap/170 '62	B Lee '40	B Gt Lakes /1739 '54	B Md/1 '59
1	Aqueous	313	<1	—	56	6	3	—	—	9	—
			41	—	128	4	4	—	—	52	—
"	Adj 65	"	<1	—	548	158	97	—	—	97	—
			41	—	832	274	181	—	—	478	—
2	Aqueous	125	<1	—	32	11	1	—	—	11	—
			40	—	6	6	1	—	—	34	—
"	Adj 65	"	<1	—	208	97	39	—	—	128	—
			40	—	208	239	45	—	—	1024	—
3	Aqueous	500	<1	—	9	2	1	—	—	23	—
			30	—	6	6	1	—	—	34	—
"	Adj 65	250	<1	—	316	181	23	—	—	416	—
			30	—	338	147	39	—	—	832	—
"	"	125	<1	—	388	119	13	—	—	194	—
			30	—	97	79	49	—	—	587	—
115	Aqueous	300	<1	1	42	5	—	20	6	—	8
			19	1	85	2	—	16	15	—	3
116	Adj 65	"	<1	85	724	128	—	316	208	—	194
			19	21	1096	274	—	256	128	—	181
156	Aqueous	500	<1	—	—	—	—	52	—	—	23
			7	—	—	—	—	42	—	—	23
140	Adj 65	250	<1	—	—	—	—	891	—	—	446
			7	—	—	—	—	1638	—	—	1260

* Except for initial tests of lots 1 and 2 (0.25 ml) the vaccine dose was 0.5 ml.
† Total CCA for all components of the vaccine.

stored vaccines was not significantly different indicating that all of the lots retained the same lack of toxicity which was displayed initially.

The growth suppression assay is based on observations for slowed gain in weight in 8-10 g mice inoculated intraperitoneally with 0.25 ml of vaccine compared with similar uninoculated control mice. Reference vaccines which have been found satisfactory in tests in animals and in man are always included for control purpose. The weight gain patterns in test mice noted on days 0, 7 and 14 for 6 vaccine lots are shown in Table III. The values obtained with the control mice were not included in the Tables since they approximated those obtained with the test materials. The growth patterns in the mice given fresh vaccine or vaccine stored up to 41 months were not disturbed indicating that all the lots retained the lack of toxicity which was displayed initially.

Discussion. Even though sensitization to egg allantoic protein has not been encountered with adjuvant 65 in man(3), it remains desirable to restrict the amount of extraneous egg protein to the minimum. By the process employed here, the virus activity/protein ratio was improved about 3-fold, from 2439 CCA units/mg protein to 6458-9298 units. Tests carried out here with ordinary commercial Sharples influenza vaccines of diverse origin usually display a value of 1000 to 3000 CCA units/mg protein. The preparation used in the present study was highly purified and water-clear. Further manipulation of purification procedure has since made it possible to attain levels of 10-15,000 CCA units/mg protein with regularity.

The antibody responses in guinea pigs to the purified aqueous vaccine compared favorably with those to ordinary Sharples vaccine. The enhancement of antibody response to the purified viral antigen by incorporation in ad-

TABLE III. Table Showing Failure of Adjuvant 65 Influenza Vaccines to Develop Toxicity on Long-Term Storage as Measured in Guinea Pigs and in Mice.

Vaccine tested				Avg thickness (64th inch) of folded skin at inj sites, according to day					Avg body wt (g) according to day		
Composition											
Lot No.	No. strains in vaccines	Total CCA units of viral antigen/0.5 ml	Age at time of test (mo)	0	1	7	10	14	0	7	14
1 (C-2780)	4	313	<1	—	7.4	12.7	12.1	9.0	8.7	14.2	21.7
			41	5.7	4.9	4.0	4.5	3.8	9.7	16.0	21.7
2 (C-2880)	4	125	<1	—	7.6*	11.1	10.0	8.2	8.6	14.1	21.5
			40	8.5	7.8	12.6	10.9	11,1	9.9	16.9	23.0
3 (C-3389)	4	250	<1	—	6.3	10.3	9.6	8.2	8.4	13.0	19.0
			30	11.6	9.7	14.4	11.9	8.6	9.6	15.3	20.6
3 (C-3390)	4	125	<1	—	3.7	5.2†	6.0	5.6	8.9	13.0	20.7
			30	9.1	8.8	11.0	9.9	8.6	9.8	15.8	22.1
115 (C-3848)	6	300	<1	—	8.4	13.3	11.6‡	10.2	9.0	12.0	20.8
			19	8.9	9.3	15.1	10.8	9.5	9.9	15.8	22.5
140 (C-4108)	2	250	<1	—	11.0	15.0	14.8	10.8	8.6	13.6	21.9
			7	11.2	8.4	13.0	13.1	9.7	9.8	15.4	22.6
Control	no vaccine (representative)								9.9	16.3	22.7

* Day 2 † Day 8 ‡ Day 11

juvant 65 was clearly shown also. The magnitude of difference in response to aqueous compared with adjuvant vaccine was greatest following the first dose of vaccine. The second dose of aqueous vaccine appeared to increase the antibody titers toward the maximally attainable level in guinea pigs while a second dose of adjuvant showed no effect beyond that which follows a single vaccine dose. The following report(6) presents data concerning tests in man which demonstrate essentially identical serologic responses to the 2 aqueous vaccines in contrast with remarkable enhancement in titer by use of adjuvant.

Two important requisites for a practicable adjuvant are stability of potency on storage and retention of safety from the standpoint of toxigenicity. The present studies demonstrated the satisfactory quality of the adjuvant 65 influenza vaccines for both requirements.

Summary. The development and animal testing of a bivalent influenza virus vaccine in immunologic adjuvant 65 employing a new highly purified viral antigen is described. The enhancement of serologic response to the highly purified viral antigen in adjuvant was as great as achieved heretofore with ordinary Sharples or protamine-concentrated vaccines used with the adjuvant. Influenza vaccines in adjuvant 65 which were stored at 4°C for periods from 9 to 41 months retained their full antigenic potency and did not acquire toxicity as measured in the guinea pig dermal and mouse peritoneal toxicity assays. The new purified vaccine offers an advantage in reducing the amount of extraneous egg allantoic protein in the vaccine thereby affording less chance for allergic sensitization.

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