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New Metabolizable Immunologic Adjuvant* for Human Use. 2. Short-term Animal Toxicity Tests. (29296)

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Other reports(1,2) in the present series describe the development of a new immunologic adjuvant, called adjuvant 65, and the results of tests of polyvalent influenza virus vaccine incorporated in a water-in-oil emulsion of the adjuvant. The adjuvant proved highly effective in potentiating antibody responses to influenza virus antigen in animals and in human beings and was found to be innocuous when given to man. Adjuvant 65 shows promise of meeting the present urgent need for a safe and effective adjuvant for enhancing immunologic responses to killed viral and other vaccines and possibly also for treating allergenic disease. The present communication describes the results of tests in animals to investigate and to compare the toxicity of the individual components of adjuvant 65 with those of Freund's incomplete mineral oil adjuvant and of killed influenza virus vaccines emulsified in adjuvant 65 with those of the same vaccines in Freund adjuvant.

Materials and methods. Adjuvant 65 consists of peanut oil, aluminum monostearate and mannide monooleate (Arlacel A). Freund's incomplete mineral oil adjuvant consists of light mineral oil and mannide monooleate. For both adjuvants, the aqueous vaccine is incorporated in the adjuvant to give a water-in-oil emulsion.

* Adjuvant 65.

Toxicity assays. Guinea pig dermal irritancy tests. Such tests of the individual components of the adjuvants and of the emulsified adjuvant vaccines were carried out, insofar as possible, in accord with the Tentative Draft, Minimum Requirements: Influenza Virus Vaccine, Emulsified in Mineral Oil, U. S. Dept. of Health, Education and Welfare, dated Jan. 30, 1956. In the tests, groups of 6 adult albino guinea pigs of Hartley strain, usually weighing 400-500 g were inoculated intradermally with the test material in 0.1 ml amounts into 6 sites. The animals were observed for 14 days during which time the thickness of the fold of skin containing the injection site was measured with a caliper and any change such as necrosis or sloughing of the skin was noted. Standard toxic and nontoxic reference preparations for control purpose referred to in the Tentative Minimum Requirements were not available. *Mouse peritoneal irritancy tests.* These were performed in general accord with the Requirements referred to above. Groups of 10 white Swiss mice of the ICR/Ha strain were used. The mice weighed 8-12 g but the individual animals selected for use in any particular test did not exceed 2.5% variation in weight. The mice were weighed prior to injection and on days 2, 3, 7 and 14 thereafter. Irritancy was expressed in terms of suppres-

sion of normal or expected weight gain for the average of the group compared with that of suitable controls. *Rabbit intramuscular irritancy tests.* The test preparations were injected on one occasion into separate areas of sacrospinalis muscle of rabbits in volumes of 0.25 or 0.50 ml. In one instance, noted in the text, repeat injections of the materials were given. The animals were sacrificed at varying time periods following treatment and injection sites in the sacrospinalis muscles were excised and sectioned serially for gross evaluation of the tissue reaction. Representative sections of injection sites were fixed with 10% formalin, embedded in paraffin, sectioned, stained with hematoxylin-eosin and examined microscopically. *Monkey intramuscular irritancy tests.* In a single experiment, pairs of rhesus monkeys weighing 3-5 lb each were injected intramuscularly into the posterior thigh muscle of one leg and 30 days later into the opposite leg with 0.5 ml amounts of aqueous influenza virus vaccine or of the same virus vaccine emulsified in adjuvant 65 or in Freund's incomplete mineral oil adjuvant. The animals were sacrificed and autopsied 6 months after the first injection. Gross and microscopic examination of the injected muscles was made as described for the rabbits above.

TABLE I. Vaccine Lots and Content of Influenza Viral Antigens.

Kind	Lot No.*	CCA units of antigen per 0.5 ml						Total CCA per 0.5 ml
		A2/Japan /305/57	A1/AA/ 1/57	A/PR8/34	A/Swine /29	B/Gt. Lakes /1739/54	B/Lee/40	
Adjuvant 65	1-C2780	125	62.5	62.5		62.5		313
Mineral oil adj.	71403	200	100	100	100	100	100	700
Aqueous	2-C2881	50	25	25		25		125
Adjuvant 65	2-C2880	50	25	25		25		125
Aqueous	3-C3388	200	100	100		100		500
Adjuvant 65	3-C3389	100	50	50		50		250
" "	3-C3390	50	25	25		25		125
" "	3-A*	200	100	100		100		500
Mineral oil adj.	3-B*	100	50	50		50		250
Mineral oil adj.	PD 057231-A	200	100	100	100	100	100	700

* The aqueous vaccine used to prepare these lots was the same as lot 3-C3388.

Vaccines. The identification and viral antigenic composition of the various influenza virus vaccines tested are given in Table I. Viral antigen content is expressed in terms of chick cell agglutination (CCA) units per 0.5 ml of aqueous or emulsified vaccine. All the viral antigens were prepared by ordinary commercial procedures. The viral antigens in lots 1-C2780 and 71403 were prepared by precipitation with protamine followed by elution (protamine-eluate). All other antigens were prepared by the Sharples centrifugation method. Lot PD-057231 A, which was prepared elsewhere for human immunization purpose, was kindly furnished by Dr. Fred Davenport and was used as a control in the present study.

Results. Guinea pig dermal irritancy tests. Representative findings in tests of adjuvant components and in tests of aqueous and adjuvant vaccines are presented in Table II. The peanut oil, mannide monooleate and aluminum monostearate components of adjuvant 65 caused only minimal inflammatory responses as evidenced by skin thickness. Mineral oil, which is ordinarily irritating upon parenteral injection, caused greater induration than did the adjuvant 65 components. The emulsified influenza virus vaccines

TABLE II. Representative Findings in Dermal Toxicity Tests in Guinea Pigs.

Materials tested	Avg thickness (64th's inch) of folded skin at inj sites, according to day													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Components														
Mineral oil (Q78)	7.7	7.7	—	—	10.2	10.7	11.4	11.7	11.8	—	—	12.3	—	11.5
Peanut oil (25600-2216)	3.7	3.7	4.1	4.4	—	—	4.5	4.7	5.2	4.8	4.7	—	—	5.5
Mannide monooleate (44B-79641)	4.5	4.3	4.7	4.8	—	—	5.2	5.7	6.0	5.9	6.2	—	—	6.3
Aluminum monostearate (2% in peanut oil)	4.8	4.6	—	—	5.1	5.6	6.1	5.9	—	—	—	5.6	5.9	6.1
Influenza vaccines														
Aqueous														
(2-C2881, 125 CCA/.5 ml)	—	4.8	4.8	5.2	—	—	5.1	—	5.0	4.7	4.7	—	—	4.9
(3-C3388, 500 CCA/.5 ml)	5.9	5.5	—	—	5.2	5.3	6.5	5.5	—	—	—	4.9	4.7	5.1
Adjuvant 65														
(1-C2780, 313 CCA/.5 ml)	7.4	7.2	8.2	—	—	10.4	12.7	12.6	12.6	12.1	—	—	10.9	9.0
(2-C2880, 125 CCA/.5 ml)	—	7.6	9.1	9.8	—	—	11.1	—	9.9	10	9.0	—	—	8.2
(3-C3389, 250 CCA/.5 ml)	6.3	6.9	7.8	8.2	—	—	10.3	10.8	11.0	9.6	10.1	—	—	8.2
(3-C3390, 125 CCA/.5 ml)	3.7	4.2	4.2	4.1	—	—	—	5.2	5.8	6.0	6.2	—	—	5.6
Mineral oil														
(PD 057231-A, 700 CCA/.5 ml)	10.0	11.4	—	—	18.1	20.6	20.3	20.7	19.7	—	—	15.8	—	16.9
(M 71403, 700 CCA/.5 ml)	11.0	12.7	—	—	17.3	17.4	16.7	15.6	14.6	—	—	11.4	—	11.1

caused a greater dermal reaction than did either the aqueous or the adjuvant components. Such reaction was generally greater with mineral oil adjuvant vaccines than with comparable adjuvant 65 vaccine. The content of the viral antigens of the mineral oil vaccines tested in guinea pigs was somewhat greater than that of the adjuvant 65 vaccines but this difference appeared unimportant in view of the greater irritancy displayed by the mineral oil alone and in the findings of tests in monkeys with lots 3 A and 3 B (see below) in which the adjuvant 65 vaccine contained twice as much viral antigen as did the mineral oil preparation.

Mouse peritoneal irritancy tests. (Table III). Mice given adjuvant components or administered adjuvant vaccines all showed increase in weight consistent with that expected for uninoculated mice and, hence, dis-

TABLE III. Representative Findings in Peritoneal Toxicity Tests in Mice.

Materials tested	Avg body wt (g), according to day					
	0	2	3	7	14	Surv./total
Components						
Mineral oil* (Q78)	—	23.4	38.2 (5 day)	38.6	48.7	
Peanut oil (25600-2216)	8.3	9.3	10.0	13.9	21.1	10/10
Mannide monooleate (44B-79641)	8.7	9.4	10.4	14.9	21.0	10/10
Aluminum monostearate (2% in peanut oil)	8.8	9.9	10.5	14.0	21.7	10/10
Influenza vaccines						
Aqueous						
(2-C2881, 125 CCA/.5 ml)	8.9	9.7	10.9	14.5	21.3	10/10
(3-C3388, 500 CCA/.5 ml)	8.7	9.8	10.2	13.3	21.4	10/10
Adjuvant 65						
(1-C2780, 313 CCA/.5 ml)	8.7	9.3	10.0	14.2	21.7	9/10†
(2-C2880, 150 CCA/.5 ml)	8.6	9.2	10.2	14.1	21.5	7/10†
(3-C3389, 250 CCA/.5 ml)	8.4	7.9	8.4	13.0	19.0	9/10†
(3-C3390, 125 CCA/.5 ml)	8.9	8.5	8.9	13.0	20.7	10/10
Mineral oil						
(PD 057231-A, 700 CCA/.5 ml)	9.1	8.9	9.7	12.1	17.4	10/10
(M 71403, 700 CCA/.5 ml)	9.2	8.9	9.8	13.0	18.9	10/10

* Earlier tests done in large mice.

† Deaths were from nonspecific cause.

TABLE IV. Gross Tissue Response in Sacrospinalis Muscle of Rabbits Given Influenza Virus Vaccine in Adjuvant 65 or in Freund's Mineral Oil Adjuvant.

Vaccine given (.25 ml)	Gross tissue response* at individual sites				
	1 day	7 days	14 days	28 days	60 days
Adjuvant 65 (Lot 1-C2780)	1+, 1+, 1+, 1+, 2+, 2+	1+, 1+, 1+, 2+, 2+, 2+	1+, 1+, 1+, 2+, 2+, 2+	±, ±, ±, 1+, 1+, 2+	±, ±, 1+, 1+
Mineral oil (Lot 71403)	2+, 2+, 2+, 2+, 2+, 2+	2+, 2+, 2+, 3+, 3+, 3+	2+, 2+, 2+, 2+, 3+, 3+	2+, 2+, 2+, 2+, 2+, 2+	2+, 2+, 2+, 2+

* 0, no lesion; ±, trace; 1+, very slight; 2+, slight; 3+, moderate reaction.

played no evident toxicity.

Rabbit muscle irritancy tests. Lot 1-C2780 adjuvant 65 and lot 71403 Freund mineral oil adjuvant influenza vaccines. Each of 15 rabbits was injected into 2 sites in the sacrospinalis muscle with 0.25 ml of adjuvant 65 vaccine and into 2 separate but similar sites with equal volumes of mineral oil adjuvant vaccine. Three animals were sacrificed on the 1st, 7th, 14th and 28th days and 2 animals on the 60th day after being given the test materials. Inoculation sites were examined grossly and microscopically. Degree of gross tissue inflammation was graded according to tissue response at each injection site as shown in Table IV. The general pathologic descriptions are as given below.

Day 1. Gross. Slight or very slight tissue changes were noted at injection sites of both vaccines. However, sites which received mineral oil were more prominent and exuded more oil on sectioning than did adjuvant 65 sites. *Microscopic.* The reactions in *mineral oil* sites resembled that following the injection of mildly irritating materials and were graded from slight to moderately severe. Muscle cell necrosis with a diffuse heterophil leukocytic infiltration, edema and a small amount of proteinaceous precipitate was noted in the central portion of the injection sites. Small cystic areas, presumably due to the oil, were present and these were outlined by a thin layer of connective tissue which sometimes invaginated into the cystic areas. The cystic areas were surrounded by a marked heterophil leukocytic infiltration. Few mononuclear cells were observed and plasma cells were seen rarely. Reactions following *adjuvant 65* vaccine were qualitatively similar to those following mineral oil vaccine but were quantitatively less marked.

Day 7. Gross. The mineral oil injection sites showed slight to moderate tissue responses and oozing of whitish material from the cut section. The adjuvant 65 sites showed very slight to slight tissue responses but no oozing from the cut section. *Microscopic.* The histomorphologic picture in the mineral oil sites was one of beginning repair but with evidence of continued irritancy and increased tissue reactions. Fibroblastic proliferation was evident and cyst formation had increased but the capsules were poorly developed. Scattered deposits of "calcific" material were noted and small foci of necrotic leukocytes and cellular debris were evident. The absolute and relative number of heterophil leukocytes was reduced and the number of lymphocytes and plasma cells was slightly increased. Macrophages and giant cells were seen frequently. The severity of the lesions in the *adjuvant 65* injection sites was less than for mineral oil vaccine. Recovery from initial irritancy was nearly complete and encapsulation of the cysts had progressed although the capsules were poorly delineated from the surrounding new connective tissue. The heterophil leukocyte response was decreased more than for mineral oil and foci of leukocytic necrosis were smaller and less frequent. Lymphocytes, macrophages, giant cells and plasma cells appeared to be more numerous than in the mineral oil sites.

Day 14. Gross. There were slight to moderate tissue reactions at the sites of mineral oil injection and only very slight to slight reactions at adjuvant 65 sites. Whitish material continued to ooze from the cut sections of the mineral oil sites and white nodules were present throughout the sites. *Microscopic.* In relation to day 7, the *mineral oil* vaccination sites showed an increase in the

number of giant cells, macrophages, lymphocytes, and plasma cells and some lymphoid aggregations were noted. The number and size of areas of leukocytic necrosis were diminished. The capsules of the cystic areas were well developed. Muscle cell necrosis was still evident. Sites which received *adjuvant 65* vaccine showed increases in numbers of mononuclear and giant cells and lymphoid aggregations and the thickness of cyst walls was increased.

Day 28. Gross. Injection sites of both vaccines showed a decrease in severity compared with that seen on day 14. *Microscopic.* Injection sites of *mineral oil* vaccine showed good recovery. Cyst capsules were well developed, heterophil leukocytes were decreased, plasma cells and diffuse and aggregate lymphocytic infiltration were increased. *Adjuvant 65* sites showed a well advanced stage of tissue repair. The cysts had thin capsules and appeared to be of smaller size. Macrophages were still present but lymphocytes and plasma cells were decreased in number, and lymphoid aggregations were found only occasionally.

Day 60. Gross. Mineral oil vaccine sites showed firm white nodules but otherwise only a slight tissue response. Sites injected with *adjuvant 65* material showed only a trace to very slight tissue response and total absence of nodules. *Microscopic.* The *mineral oil* sites showed that the pathologic process was nearly static and consisted mainly of small encapsulated cysts, a few giant cells, and minimal mononuclear infiltration. However, a few foci of heterophil leukocytic infiltration could be found. Areas of lymphocyte aggregates were frequent. Plasma cells were found in the lesions and particularly in the aggregates of lymphocytes. *Adjuvant 65* sites consisted mainly of thinly encapsulated cysts with lymphocyte, plasma cell and macrophage infiltration. There was no evidence of active irritancy.

Lot 2-C2880 influenza virus vaccine in adjuvant 65. Nine rabbits were each injected in 4 different areas of the sacrospinalis muscles with 0.5 ml of the vaccine. Two rabbits were sacrificed at each time period of 1, 7, 14 and 28 days and one rabbit was sacrificed 60 days following injection. All sites were examined at autopsy. The volume of material injected into each site was twice that of the previous experiment resulting, as would be anticipated, in an increase in amount of tissue response. The *gross* tissue responses were recorded as very slight to slight on day 1, slight to moderate on day 7, very slight to slight on day 14, very slight to slight on day 28, and trace to slight on day 60. The major difference in gross appearance from that noted in the previous experiment was the presence of oily material which exuded from the cut surfaces on days 1, 7 and 14. This appeared to be related to the larger dose volume. *Microscopic.* The tissue changes were qualitatively similar to those noted above for adjuvant 65 vaccine but were quantitatively greater because of the larger volume injected. The same type of encystment of oil and the same patterns for leukocytic changes were present as noted in the previous experiment. There was no evidence of abscess formation.

Lot 3-C3388 (500 CCA) aqueous, lots 3-C3389 (250 CCA) and 3-C3390 (125 CCA) adjuvant 65 influenza virus vaccines. These vaccines were tested as described above except that each animal received initially 0.5 ml of vaccine into the cephalad portion of each sacrospinalis muscle. Thirty days later, a second 0.5 ml dose was given into the original injection site on one side and 0.5 ml was injected one inch caudad to the original injection site on the opposite side. Groups of 20 rabbits were used for each vaccine and sacrifice for observation purpose was made on days 7, 30, 37 and 60 following the first dose of material. The reactions to the *adjuvant 65* vaccines were similar to those noted above. Worthy of note, reinjection into the original site did not increase the reaction beyond that seen in the sites which had not been reinjected and a second injection into a new site did not cause a "flare-up" of tissue reaction at the original injection site. Following *aqueous* vaccine, gross examination showed only trace or negative tissue reaction. Microscopic examination revealed trace to very slight tissue responses which were generally in an advanced stage of repair by the seventh day. No cyst formation was noted.

Monkey muscle irritancy tests. Pairs of 3 to 5 lb rhesus monkeys were each injected intramuscularly into one posterior thigh with 0.5 ml of adjuvant 65 influenza vaccine lot 3-A (500 CCA), lot 3-B (250 CCA) mineral oil adjuvant, or lot 3-C3388 (500 CCA) aqueous vaccine. A second dose was given into the opposite leg one month later. The animals were sacrificed 6 months following the initial injection and the sites were examined grossly and microscopically. *Mineral oil adjuvant.* Injection sites were observed grossly in one leg of one monkey and in both legs of the second animal. Lesions which measured as much as 3 × 1.5 × 1 cm were noted. Pus-like material was noted in at least one injection site. *Microscopically,* the lesions due to mineral oil adjuvant vaccine were as striking as those observed grossly. Of 5 muscle sections from the 4 injection sites examined, one showed minor tissue changes consisting of small encapsulated oil cysts and slight leukocytic infiltration consisting primarily of lymphocytes, macrophages and a few plasma cells in the connective tissue surrounding the cysts. Two other sections showed small but heavily encapsulated areas in which there were large numbers of leukocytes with an admixture of giant cells and lymphocytes and areas of necrotic leukocytes. The remaining 2 sections revealed similar large areas which exhibited necrotic centers of considerable magnitude. Thus, abscess formation was observed. *Adjuvant 65. Gross* examination showed, in one site, pinhead sized white foci which resembled parasitic cysts rather than the result of treatment. *Microscopic* examination was made of 6 sections from the 4 injection sites of the 2 animals. Two sections revealed very slight tissue reactions which consisted of collections of macrophages located generally in the cleavage planes of the muscles. There was no evidence of scar formation and the adjacent muscle fibers were normal. Polymorphonuclear cells were seen only rarely and infrequent lymphocytes and plasma cells were noted. In contrast to mineral oil vaccine, there was no evidence of potential abscess formation. There was no evidence of residual tissue reactions in the other 4 sections examined. One section showed foci of parasitic encystation. *Aqueous.* Gross and microscopic examination of the muscles into which the aqueous vaccine had been injected did not reveal evidence of tissue reaction related to treatment.

Discussion. Freund's incomplete mineral oil adjuvant with influenza virus vaccine has enjoyed extensive experimental use in human subjects for more than a decade(4). Additionally, the adjuvant has been evaluated for use with poliovirus vaccines(5-7) and has been employed for increasing therapeutic responses to allergenic substances in human beings(8,9). The safety of mineral oil adjuvant has been questioned, however, on the basis of experimental data in animals which indicates that mineral oil alone or Freund's complete or incomplete mineral oil adjuvant given with or without added antigens may cause or be associated with induction of plasma cell tumors, autoimmune reactions, or with excessive formation of focal granulomata(3,10-19). These effects are likely without any significance in the human being since the only adverse effects noted to date for man have been local reactions at the injection site and apparent increase in sensitivities in inoculated persons(5,20-21). A principal objection to Freund adjuvant is the apparent long-term persistence of the mineral oil(3) in the body. It was considered important to seek an effective adjuvant comprised of more readily metabolizable substances and adjuvant 65 was developed to meet this objective.

A principal difficulty encountered with Freund adjuvant in the early period of its use was in the toxicity of certain lots of mannide monooleate which contained excessive amounts of free oleic acid and which was the apparent cause for the formation of sterile abscesses in a portion of recipients of the vaccine. This led to stringent tests for toxicity of the components of the adjuvant such as are described in the Tentative Draft, Minimum Requirements: Influenza Virus Vaccine, Emulsified in Mineral Oil, U. S. Dept. of Health, Education and Welfare, Nat. Inst. Health, dated Jan. 30, 1956. These same tests for irritancy in guinea pigs and in

mice have been applied with equal success to the components of adjuvant 65. Standard toxic control materials such as designated in the tentative Requirements were not available and were found not to be necessary. However, a control preparation previously found to be acceptable was always included in each test and the component being assayed was considered satisfactory if the findings showed no appreciable deviation from those obtained with the controls.

Although not designated in the Tentative Requirements, the guinea pig dermal and the mouse intraperitoneal tests have been applied to the emulsified vaccines themselves as an added guide for toxicity. The reactions to the emulsified vaccines were greater than for the individual adjuvant components alone and, in addition, the mineral oil adjuvant vaccines always caused far more reaction than did the adjuvant 65 formulations.

While the mouse and guinea pig tests served as a guide for toxicity and safety, chief reliance was placed on the rabbit muscle test for irritancy. The sacrospinalis muscle of the rabbit is commonly used to evaluate irritancy of materials to be administered parenterally. This muscle is very dense compared with other muscles, *e.g.*, the gluteus muscles of man. Hence, the degree of diffusion of material is limited and the degree of tissue reaction is far greater than would be obtained in other muscles. The pathologist must take cognizance of this fact, therefore, in judging reactions since substances which cause relatively severe reactions in the sacrospinalis may cause only a mild reaction in muscles which are not so dense. The evaluation of safety in the muscle irritancy test is based not only upon the immediate irritant effect but also upon the continued presence of inflammatory processes for long periods. The great sensitivity of the sacrospinalis muscle test is evidenced by the fact that such innocuous material as physiological saline solution can be shown microscopically to cause very slight tissue reactions within 24 hours with recovery in 4 to 7 days. The findings observed in monkeys given adjuvant 65 vaccine or mineral oil vaccine generally resembled those that might be expected in rabbits if examined longer than 60 days after injection. The greater toxicity of the mineral oil preparation was rather spectacularly demonstrated by the formation of abscesses in the monkey.

The initial pathologic response to adjuvant 65 influenza virus vaccine may be described as a reaction to a mildly irritating foreign substance with resultant acute inflammation. The acute reaction became subacute after several days at which time there was a substantial decrease in polymorphonuclear leukocytes (heterophils) and an increase in lymphocytes, plamsa cells and macrophages. By the twenty-eighth and continuing through the sixtieth day, the macrophage was the predominant infiltrating cell. There was only slight cell necrosis and the oily adjuvant material was gradually removed, presumably by phagocytosis, with progressive resolution of the tissue damage. Although oil cyst formation did occur, this decreased with time and there was no significant formation of scar tissue. Additionally, the vaccine failed to induce any apparent sensitivity to itself as judged by tissue responses to a second dose of the vaccine at the same or a distant site.

Mineral oil adjuvant vaccine, by contrast, caused a more severe reaction. The basic pathologic features were the same as for adjuvant 65 vaccine but the degree of acute and subacute inflammatory response to the mineral oil vaccine was greater and more protracted. It appeared that the oily material in the mineral oil adjuvant which was retained at the injection site caused continued irritancy which could lead to scar and abscess formation. The extent to which adjuvant 65 and mineral oil formulations are removed from the original site and cause anatomical alterations at distal sites is being sought in additional experiments. It seems reasonable to judge, however, that adjuvant 65 is metabolized completely and is unlikely to cause damage in other organs.

While prolonged retention of antigen and the evoking of a suitable inflammatory response at the injection site apparently are requisites for adjuvant activity(3,4), there is a necessity to limit the resultant pathologic processes to an inconsequential clinical

level. Hence, the desire for enhanced antibody response must always be tempered with a consideration of the degree of clinical reaction. The available data, shown in papers 1 and 3(1,2) of this series, suggests that influenza vaccine in adjuvant 65 gives about the same degree of immunologic enhancement as does mineral oil adjuvant. The bonus in terms of milder clinical reaction and the greater metabolizability of the constituents of adjuvant 65 is a positive value which, on theoretical bases at least, provides an advantage for use in the human being (1,2). Studies to measure possible long-term pathologic effects attending use of adjuvant 65 in animals are in progress.

In developing the adjuvant 65 preparation, due care was taken to use peanut oil which was free of antigenic substances which might cause hypersensitivity reactions. In connection with these studies, Ouchterlony gel diffusion tests were carried out using aqueous extracts of green peanuts, crude peanut oil, or U. S. P. peanut oil and antisera prepared against each of these substances in rabbits. It was found (not reported herein) that aqueous extracts from green peanuts gave 4 to 7 precipitin bands in tests with antiserum against green peanuts. The crude as well as the U. S. P. peanut oils failed to give bands in tests with all sera. Additionally, the aqueous green peanut extract gave no reactions with sera from animals injected with crude or U. S. P. peanut oil. These findings indicate that the peanut oils were devoid of water soluble antigens which could be detected by the Ouchterlony precipitin test method but do not preclude the possibility of an immune response.

Summary. A new immunologic adjuvant, called adjuvant 65, was assayed for toxicity, safety and irritancy in several species of animals. Clinical, gross and histomorphologic examinations were made. The reactions which resulted from adjuvant 65 vaccines were mild and inconsequential and far less than were obtained in comparable tests with Freund's incomplete mineral oil adjuvant vaccine. The comparative pathology for the 2 different kinds of adjuvant is presented and the theoretical and practical advantages of adjuvant 65 are discussed.

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