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New Metabolizable Immunologic Adjuvant* for Human Use. I. Development and Animal Immune Response. (29295)

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A principal purpose for employment of an immunologic adjuvant is to achieve a more durable immunity of a higher level employing a smaller antigenic mass in a fewer number of doses than could be achieved by administration of the equivalent aqueous antigen. Development of an immunologically satisfactory and pharmacologically acceptable adjuvant seems a prime essential to preparation of workable multivalent killed virus vaccines such as are required to be effective and practical against the viral respiratory disease complex. Freund's incomplete mineral oil adjuvant without added Mycobacteria(1) has enjoyed widespread use in experimental vaccines. Although highly satisfactory from the standpoint of achieving persistent high level of antibody following immunization(1-6), routine application of Freund's adjuvant to man has not been achieved to date likely because of a large body of data relating to induction of potentiation of plasma cell tumors in BALB/c mice, of induction of autoimmune reactions, and of formation of disseminated focal granulomata in animals injected with mineral oil alone or with complete or incomplete adjuvant with or without added antigens(1,7-16). These effects found in animals are likely without significance in the human being since studies in man have indicated no significant adverse effect referable to the adjuvant other than local reactions at the injection site(17) or some apparent increase in hypersensitivities in inoculated individuals(18,19). Perhaps the principal objection to Freund mineral oil adjuvant acceptance, albeit theoretical, is the apparent long-term persistence of the mineral oil(1). It seemed important, therefore, to seek an effective adjuvant which would be free of saturated aliphatic hydrocarbons.

Work conducted in these laboratories since 1958‡ has been directed to development of a safe and effective immunologic adjuvant for human use which would be comprised of readily metabolizable components which fail to give evidence of significant adverse effects in the human and in animal species. These investigations culminated in development of a stable water-in-oil preparation which is called adjuvant 65 and which comprises aqueous antigens in peanut oil, aluminum monostearate, and mannide monooleate (Arlacel A). Each of the components of the adjuvant enjoys the precedent of long-term use in the human species(1-6,20-24). Additionally, extensive tests carried out to date of the new

* Adjuvant 65.

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‡ Earlier investigations of immunologic adjuvants were conducted in these laboratories by Drs. M. R. Hilleman, J. McKenna, J. D. Mullins, K. Stevens, T. Stim, B. Sweet, A. A. Tytell and A. F. Woodhour.

TABLE I. Vaccine Lots and Antigen Content.

Influenza virus strain	Formulation: Hexavalent — Aqueous Lot 116-C3854 (0.5 ml)	Hexavalent — Adjuvant 65 Lot 115-C3848 (0.5 ml)	Hexavalent — Mineral oil Lot 117-C3849 (0.25 ml)	Quadrivalent — Aqueous Lot 2-C2881 (0.25 ml)	Quadrivalent — Adjuvant 65 Lot 2-C2880 (0.25 ml)
A2/Japan/170/62	50 CCA*	50 CCA	50 CCA	25 CCA	25 CCA
A1/AA/1/57	" "	" "	" "	12.5 "	12.5 "
A/PR8/34	" "	" "	" "	" "	" "
A/Swine/29	" "	" "	" "	—	—
B/Md/1/59	" "	" "	" "	—	—
B/Lee/40	" "	" "	" "	—	—
B/Gt. Lakes/1739	—	—	—	12.5 CCA	12.5 CCA
Total number	300	300	300	62.5	62.5

* Chick cell agglutinating units.

adjuvant in a variety of animals and of man have given indication of a high degree of efficacy and safety. The findings in investigations of adjuvant 65 used with polyvalent influenza virus vaccine are summarized here and in subsequent reports(25,26).

Materials and methods. Vaccines. The aqueous influenza virus vaccines were polyvalent materials prepared from viruses propagated in embryonated hens' eggs and purified by the commercial Sharples centrifugation method. Inactivation of viral infectivity was by treatment with formalin. The final vaccine conformed in all respects to the Minimum Requirements, Influenza Virus Vaccine, U. S. Dept. of Health, Education and Welfare. The same aqueous vaccine lots were used to prepare comparable lots of vaccine emulsified in Freund's mineral oil adjuvant or in adjuvant 65. *Freund's mineral oil adjuvant.* Incomplete Freund adjuvant without added Mycobacteria was used(1). The aqueous vaccine was emulsified with an equal volume of sterile mixture containing 90% of light mineral oil (Drakeol 6VR, Pennsylvania Refining Co., Butler, Pa.) and 10% emulsifier (Arlacel A, mannide monooleate, Atlas Chemical Industries, Inc., Wilmington, Del.). The mineral oil and emulsifier were tested and certified in our laboratories in accordance with 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. The emulsified vaccine proved stable in the centrifuge test as described in the Tentative Draft, Minimum Requirements, and there was no evidence of breaking of the emulsion when the vaccines were stored at 37°C for at least 14 days. *Adjuvant 65.* The aqueous vaccines were emulsified with an equal volume of a sterile mixture containing 86% peanut oil (USP, XVI), 10% mannide monooleate, and 4% aluminum monostearate (USP, XVI). The mannide monooleate was tested as described above. The peanut oil and aluminum monostearate were tested for toxicity in guinea pigs and in mice in accordance with the Tentative Draft, Minimum Requirements, above, with satisfactory results. The emulsions proved stable in the same tests described above for Freund adjuvant vaccine and, additionally, were stable on storage at 4°C for at least 29 months. *Influenza viral antigen content of vaccines.* Table I summarizes the viral antigen content of the several vaccines tested in the present study. The quadrivalent vaccines were adjusted to contain 62.5 chick cell agglutinating (CCA) units of antigen per 0.25 ml. The hexavalent vaccines contained 300 CCA per 0.5 ml of aqueous or adjuvant 65 vaccine or per 0.25 ml of mineral oil adjuvant material. *Antibody responses in experimental animals.* Comparative tests for immunizing potency were carried out in guinea pigs, mice, rabbits and monkeys. The vaccination regimens and bleeding schedules were as stated in the Figures and in the text. Serum samples were collected aseptically and stored frozen at —20°C until tested by the hemagglutination-inhibition (HI) procedure. The HI tests were

performed by the ordinary pattern test procedure(27) using the identical strains of virus employed in the vaccines. All sera were treated with trypsin-periodate or with receptor destroying enzyme (RDE) to remove nonspecific inhibitors(28,29). The lowest serum dilution tested was 1:10 and all titers were expressed as initial serum dilution prior to addition of the other reagents in the test.

Results. Comparison of immunologic potentiation by Freund mineral oil adjuvant with that by adjuvant 65. Guinea pigs. Groups of ten 400 to 500 g guinea pigs were injected intramuscularly on one occasion with 0.5 ml of aqueous, 0.25 ml of mineral oil adjuvant, or 0.5 ml of 5 different preparations of hexavalent influenza virus vaccine in adjuvant 65. Antibody response was measured in terms of seroconversion to positive status and of geometric mean HI antibody titers of the individual sera for each of the homologous strains in serum samples taken one month after vaccination. The findings (Fig. 1) reveal that the serologic responses to the virus strains in the aqueous vaccine ranged from zero to 100 percent while nearly all the animals given Freund mineral oil adjuvant or adjuvant 65 vaccine developed antibody to all strains. The geometric mean HI antibody levels following aqueous vaccine were generally poor and ranged from 1:1 to 1:42 for the various strains. By contrast, both the mineral oil and adjuvant 65 preparations showed a remarkaby enhanced serologic response and the mean titers were generally 16 to 32 times as great for each strain as were

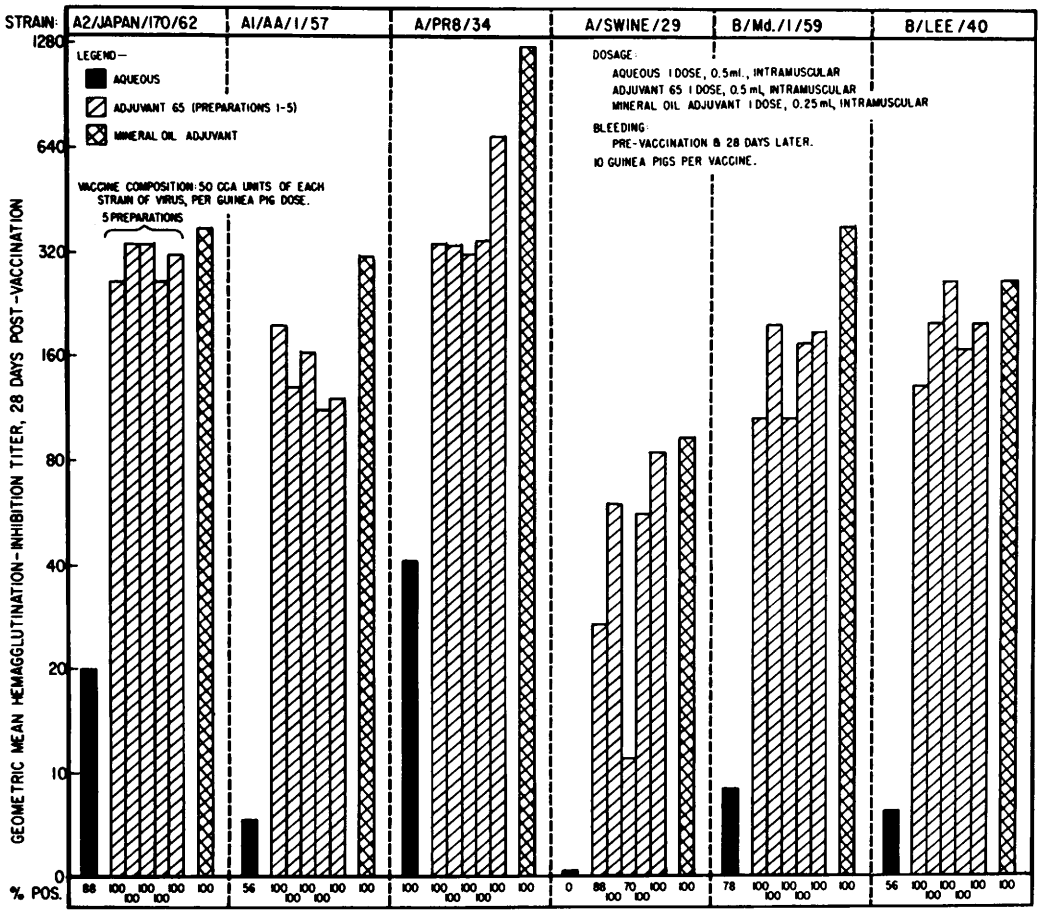

FIG. 1. Comparison, hemagglutination-inhibition antibody response in guinea pigs to one dose of aqueous, adjuvant 65, or mineral oil adjuvant hexavalent influenza virus vaccine.

obtained with the corresponding aqueous material. There was a small variation between the mean titers obtained with the 5 different preparations of adjuvant 65 vaccine. The mean titers in the guinea pigs following mineral oil vaccine most often were slightly greater than those following adjuvant 65, the former being 1.2 to 2 times as great as the arithmetic average for the 5 adjuvant 65 preparations. *Mice.* Groups of ten 18-20 g mice were inoculated subcutaneously on one occasion with 0.2 ml of aqueous, 0.1 ml of mineral oil or 0.2 ml of the 5 different preparations of adjuvant 65 hexavalent influenza virus vaccine. Antibody response was expressed as arithmetic mean HI antibody titer of equal volume pools of sera collected 42 days after vaccination. Fig. 2 shows that the mice injected with aqueous vaccine responded only to the PR8 strain. By contrast, animals which received mineral oil or adjuvant 65 vaccines developed antibody against all strains with mean titer values ranging from 1:20 to 1:640. The mean antibody titers of sera from animals which received adjuvant 65 vaccine were generally greater than those from animals given mineral oil adjuvant vaccine. This varied from nearly alike for B/Md/1/59 virus to a greater than 2-fold difference. The generally higher serum titers obtained with adjuvant 65 compared with mineral oil vaccine in mice were in contrast to those obtained in guinea pigs in which the responses to mineral oil adjuvant were greatest.

Long-term antibody patterns following quadrivalent aqueous or adjuvant 65 influenza virus vaccine in 3 animal species. Guinea pigs. Groups of 25 Hartley strain animals weighing 400-500 g were each given two 0.25

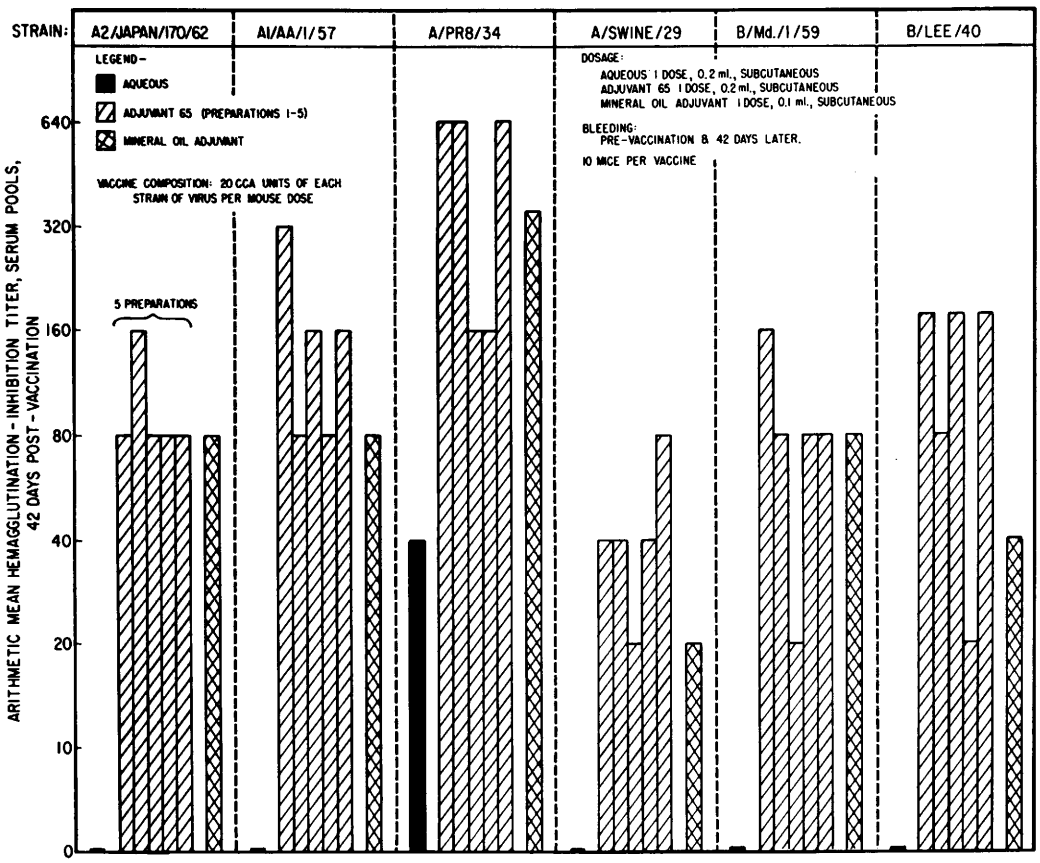

FIG. 2. Comparison, hemagglutination-inhibition antibody response in mice to one dose of aqueous, adjuvant 65, or mineral oil adjuvant hexavalent influenza virus vaccine.

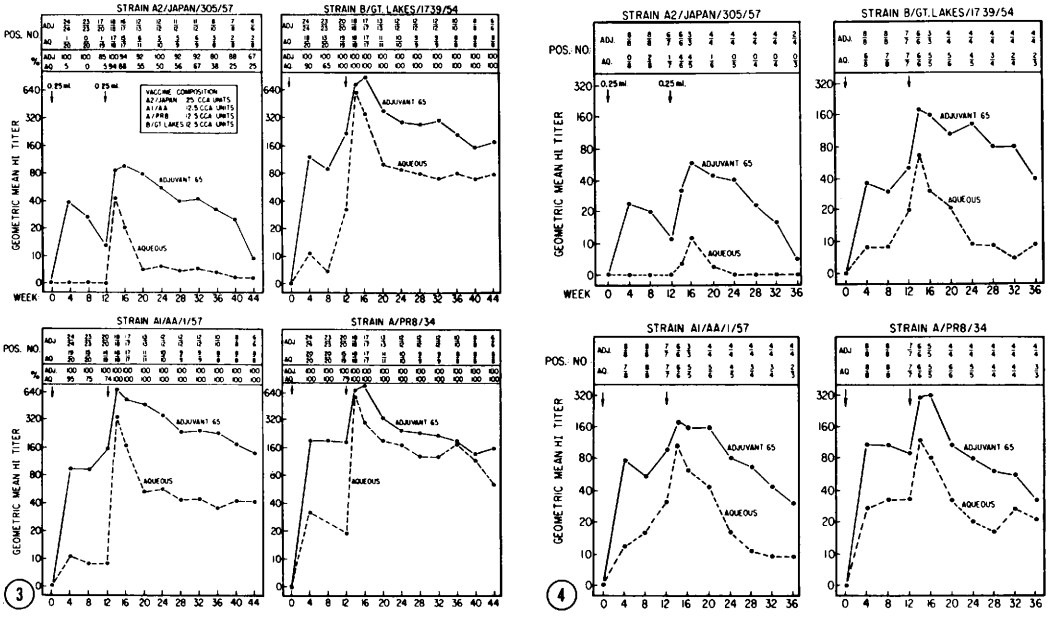

FIG. 3. Comparison hemagglutination-inhibition antibody responses in guinea pigs given two 0.25 ml intramuscular doses of quadrivalent aqueous or adjuvant 65 influenza virus vaccine 12 weeks apart.

FIG. 4. Comparison hemagglutination-inhibition antibody responses in rabbits given two 0.25 ml intramuscular doses of quadrivalent aqueous or adjuvant 65 influenza vaccine 12 weeks apart.

ml intramuscular injections of aqueous or adjuvant 65 tetravalent vaccine spaced 12 weeks apart. Blood samples were collected at 4-week intervals for a total of 44 weeks. Fig. 3 presents the findings in terms of fraction and percent of animals seropositive at a particular time period and according to geometric mean HI titer of the individual sera against each of the influenza virus strains included in the vaccine. As already shown for the hexavalent vaccine, there was a very great serologic response to a single dose of adjuvant 65 vaccine and comparatively little or no response to the aqueous preparation. A remarkable increase in titer attended administration of a second dose of either vaccine. Antibody responses against the adjuvant 65 component were developed rapidly, and there was only a slow decline in titer of antibody over the 44-week period of observation. The maximal antibody titers achieved following the second dose of aqueous vaccine were only slightly less than those following the second dose of adjuvant vaccine but the level of antibody generally declined far more rapidly and to a much greater extent after aqueous than following adjuvant 65 vaccine. *Rabbits.* Groups of 9 young adult New Zealand albino rabbits, each weighing initially 4-5 lb, were given 2 intramuscular doses of aqueous or adjuvant 65 vaccine 12 weeks apart and the serologic patterns were followed for 36 weeks. The findings in the rabbits shown in Fig. 4 generally resembled those obtained in guinea pigs. *Monkeys.* Groups of 15 rhesus monkeys weighing 3 to 5 lb were each given 0.25 ml of quadrivalent aqueous or adjuvant 65 influenza vaccine intramuscularly on one occasion and a second dose of the same material was administered one month later. Antibody patterns were followed for 44 weeks after the first vaccine dose. Fig. 5 shows that the responses to the aqueous vaccine were very poor even following the second dose and that the antibody which was elicited was lost very rapidly. By contrast, the responses to adjuvant 65 vaccine were excellent and persisted for at least 44 weeks.

Stability of antigenic potency of adjuvant 65 vaccine on storage at 4°C. Adjuvant 65 lot 1-C2780 and aqueous lot 1-C2781, not described in Table I, were assayed for po-

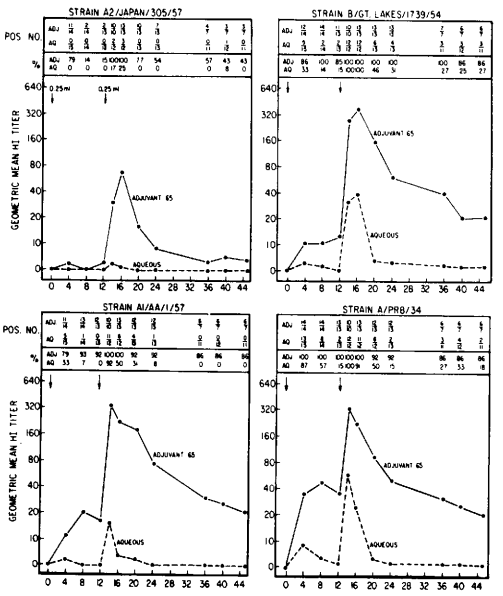

FIG. 5. Comparison hemagglutination-inhibition antibody responses in rhesus monkeys given two 0.25 ml intramuscular doses of quadrivalent aqueous or adjuvant 65 influenza vaccine 12 weeks apart.

tency at various times during a 1-year period in guinea pigs as described above. A single intramuscular dose of vaccine was given and the animals were bled 28 days after vaccination. Twelve to 21 animals were used per group in the first 2 tests and 6 or 7 animals in the last 2 tests. The influenza viral antigens were prepared by the protamine-eluate method and are described in detail elsewhere (26). The antigen content of the vaccine was 125 CCA units of A2/Japan/305/57 and 62.5 units of each of the other strains per 0.5 ml (Total 313). Both vaccines (Table II) retained their original capacity to stimulate antibody for at least one year on storage at 4°C.

Discussion. Previous attempts by other workers(30-32,35) have failed to provide aqueous emulsions of antigens in vegetable oils which fulfilled the criteria of being acceptable for use in and tested in man, which were sufficiently stable, and which gave worthwhile enhancement of immunologic response. The present report describes the development of such an adjuvant which is highly effective in potentiating the immune responses in guinea pigs, mice, rabbits and rhesus monkeys to polyvalent influenza virus antigens. By incorporating influenza virus vaccine in adjuvant 65, far greater, more consistent, and longer persisting antibody was achieved than when the corresponding aqueous vaccine was employed. Tests to measure HI antibody responses in animals 28 days following vaccination revealed that the immunologic enhancing effect of adjuvant 65 on influenza virus vaccine was roughly the same as when Freund's incomplete mineral oil adjuvant was employed.

Influenza virus vaccine in adjuvant 65 is a highly viscous water-in-oil emulsion. The emulsion is stable for at least 29 months when stored at 4°C and the antigenic potency is retained undiminished for at least one year. The precise mechanism for the action of adjuvant 65 in enhancing immunologic responses is unknown but may be presumed, as for Freund mineral oil adjuvant(33,34), to be related to retention of the antigen in emulsified form with slow continuous release of antigen and with the evoking of an inflammatory response which is appropriate for increased antibody production.

All the components of adjuvant 65, *i.e.*, peanut oil, mannide monooleate and alumi-

TABLE II. Stability of Antigenic Potency of Aqueous and Adjuvant 65 Influenza Virus Vaccines Stored at 4°C as Measured in Guinea Pigs.

	Geometric mean HI antibody titer 30 days after vaccination, according to virus and to month after manufacture															
	A2/Japan/305/57				A1/AA/1/57				A/PR8/34				B/Gt. L./1739/54			
Vaccine	0 mo*	3	5	12	0	3	5	12	0	3	5	12	0	3	5	12
Adjuvant 65 Lot 1-C2780	97	49	80	79	158	119	234	97	548	587	832	776	97	97	104	69
Aqueous Lot 1-C2781	3	1	2	1	6	3	5	4	56	69	97	79	9	11	11	9

* Length of time lapsed after manufacture and storage at 4°C.

num monostearate have been used for many years in injectables in the human species (1-6, 20-24). Penicillin with aluminum monostearate in peanut oil, *e.g.*, was widely employed at one time as a repository antibiotic (22-24). Arlacel A (mannide monooleate) has found long-term utilization as the emulsifier for Freund adjuvant in various vaccines and allergenic preparations(1-6,21).

Studies of variants of the present adjuvant 65 formulation have shown that emulsions of a variety of vegetable oils can be prepared. Additionally, adjuvant 65 or variations thereof have been used successfully (unpublished) for potentiating the immunologic responses to a variety of other monovalent or polyvalent formulations of microbes such as rhinoviruses, polioviruses, *Mycoplasma pneumoniae*, parainfluenza viruses, and respiratory syncytial agent.

The present report is limited to data relating to immunologic responses in animals and stability properties of influenza virus vaccine in adjuvant 65. Other reports(25,26) summarize the findings in gross and histomorphologic studies of adjuvant 65 in animals and of the excellent long-term potentiation of immune response in large-scale tests in human subjects without apparent untoward effects. Studies of long-term pathology in animals are in progress.

Summary. A new immunologic adjuvant, adjuvant 65, is described and the results of extensive tests of polyvalent influenza virus vaccine in the adjuvant are presented. The vaccine consists of a water-in-oil emulsion in which the adjuvant portion consists of peanut oil, mannide monooleate and aluminum monostearate. The adjuvant effect measured in terms of hemagglutination-inhibition antibody responses 28 days after vaccination in mice or guinea pigs is roughly equal to that obtained with Freund's incomplete mineral oil adjuvant. Adjuvant 65 influenza vaccine showed striking enhancement and long-term persistence of antibody in guinea pigs, rabbits and monkeys compared with the corresponding aqueous vaccines. The findings in histomorphologic-pathologic studies in animals and the clinical and serologic results obtained in large-scale studies in man are presented elsewhere. Adjuvant 65 shows promise of meeting the present urgent need for a safe and effective adjuvant for enhancing immunologic responses to viral and other vaccines and possibly also for treating allergic disease.

The authors are indebted to G. Zandle, J. Armstrong, W. P. Fisher and R. R. Roehm for valuable technical assistance.

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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-