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Hyperpotentiation by Synthetic Double-Stranded RNA of Antibody Responses to Influenza Virus Vaccine in Adjuvant 65 (33983)

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The previous reports by our group (1–11) described the development and evaluation of a new immunologic adjuvant named adjuvant 65, which was applied to killed influenza vaccine. The adjuvant preparation consisted of a water-in-peanut oil emulsion of the aqueous vaccine employing mannide monooleate (Arlacel A) as emulsifier and aluminum monostearate as stabilizer. Use of the adjuvant effected great increase in the height of antibody compared with aqueous vaccine and this differential was maintained for several years following the last dose of vaccine.

Braun and Nakano (12) demonstrated that certain oligodeoxyribonucleotides and ribonucleotide homopolymers, especially complexed double-stranded (e.g., rA:rU) molecules may function as potent stimulators of the immune mechanism. The question was raised, therefore, whether addition of complexed RNA homopolymers to adjuvant 65 might provide a potentiation of antibody response which would exceed that of adjuvant 65 or of the polynucleotides alone. It was found that complexed polynucleotides Poly I:C (rI:rC) and Poly A:U (rA:rU) effected a very marked hyperpotentiation of the antibody responses to influenza antigens. These same polymers were found previously by our group (13, 14) to serve as highly potent inducers of interferon and of protection against viral infections *in vitro* and *in vivo*. The present report presents the findings in qualitative and quantitative studies to measure the potentiation by the polynucleotides of the antibody responses to influenza viral antigens in adjuvant 65.

Materials and Methods. Vaccines: Quadrivalent, trivalent, or monovalent influenza virus vaccines containing the kinds and amounts [chick cell agglutinating (CCA) units] of formalin-killed influenza viral antigens shown in Table I were employed. All vaccines were prepared according to procedures ordinarily used for commercial influenza vaccine production. The physiochemically purified (5) and the filter-purified (8) vaccines were described previously. The vaccines were prepared in 0.15 *M* phosphate buffered saline solution, pH 7.2. Certain of the vaccines were precipitated with potassium aluminum sulfate (alum) and contained 8.0 mg of alum/ml of aqueous vaccine.

Polynucleotides. The polyinosinic (Poly I or rI), polycytidylic (Poly C or rC), polyadenylic (Poly A or rA), and polyuridylic (Poly U or rU) acid ribonucleotides which were used to potentiate immune responses (see Table I) were obtained from Miles Laboratories. The individual nucleotides were dissolved in phosphate buffered saline and sterilized by filtration through UF sintered glass. Complexes of Poly I:C (rI:rC) and of Poly A:U (rA:rU) were prepared by mixing equimolar solutions of the complementary polymers and allowing them to react at room temperature. The complexing was measured

TABLE I. Summary of Influenza Vaccine Formulations which Were Compared.

Expt. no.	Animal species	Influenza vaccine: Purification	Influenza vaccine: Virus antigen content	Influenza vaccine: CCA[a] (content/dose)	Formulations tested: Kind	Formulations tested: Polynucleotide (kind and amount; μg/dose)
1-537	Guinea pig	Physicochemical	Quadrivalent			
			A/PR8/34	200 and 50	Aqueous alone	0
			A2/Japan/170/57	100 and 25	Aqueous + Poly A:U	rA:rU 262
			A2/Taiwan/1/64	100 and 25	Aqueous + Poly I:C	rI:rC 263
			B/Mass/3/66	200 and 50	Adj 65 alone	0
				(2 different antigen levels tested)	Adj 65 + Poly A:U	rA:rU 263
					Adj 65 + Poly I:C	rI:rC 263
2-541	Monkey	Physicochemical	Quadrivalent			
			A/PR8/34	200	Aqueous alone	0
			A2/Japan/170/57	100	Adj 65 + Poly I:C	rI:rC 263
			A2/Taiwan/1/64	100	Adj 65 alone	0
			B/Mass/3/66	200		
3-544	Guinea pig	Filter-purified	Trivalent		Aqueous alone	0
			A2/Japan/170/57	150	Aqueous + Poly I	rI 260
			A2/Taiwan/1/64	150	Aqueous + Poly I	rI 130
			B/Mass/3/66	300	Aqueous + Poly C	rC 260
					Aqueous + Poly C	rC 130
					Aqueous + Poly I:C	rI:rC 260
					Alum alone	0
					Alum + Poly I	rI 260
					Alum + Poly I	rI 130
					Alum + Poly C	rC 260
					Alum + Poly C	rC 130
					Alum + Poly I:C	rI:rC 260
					Adj 65 alone	0
					Adj 65 + Poly I	rI 260
					Adj 65 + Poly I	rI 130
					Adj 65 + Poly C	rC 260
					Adj 65 + Poly C	rC 130
					Adj 65 + Poly I:C	rI:rC 260

TABLE I (continued)

Expt. no.	Animal species	Influenza vaccine			Formulations tested	
		Purification	Virus antigen content	CCA[a] (content/dose)	Kind	Polynucleotide (kind and amount; μg/dose)
4-547	Guinea pig	Filter-purified	Monovalent A2/Japan/170/57 or A2/Taiwan/1/64 or B/Mass/3/66	Titration of antigen at 100 and 10	Adj 65 alone	Each viral antigen, each concentration at 0
					Adj 65 + Poly I:C	rI:rC 260
					Poly I:C	rI:rC 26
					Poly I:C	rI:rC 2.6

[a] Chick cell agglutinating units.

by hypochromic effect in a DB-G spectrophotometer.

Formulations (see Table I). Aqueous, alum, and adjuvant 65 type vaccines were utilized in the study. In certain instances single or complexed homopolymers of ribonucleic acid were added. Adjuvant 65 vehicle was prepared as described earlier (1, 10) and emulsificiation was carried out using a Mulsichurn reciprocator (Mulsijet, Inc.). The compositions of the various formulations which were tested are given in detail in Table I. A great many additional formulations which represent quantitative variations of the above were also prepared and tested. Space limitation precludes their inclusion in the present report. However, they will be mentioned and discussed in the text.

Animals. The tests were carried out in guinea pigs or in monkeys. The guines pigs were of the Hartley strain obtained from Perfection Breeders, Douglasville, Pa., were of both sexes, and weighed from 450 to 550 g. The vaccines were given in 1 or 2 doses. All vaccines were administered by the intramuscular route. The aqueous and alum vaccines were given in 1.0-ml dose and the adjuvant 65 vaccines in 0.5-ml dose. In the studies, 6 guinea pigs or 5 monkeys were used for each vaccine.

Serology. The immune responses to vaccination were assayed in terms of height of titer of hemagglutination-inhibiting (HI) antibody. All animals were bled prior to the first dose of vaccine and at the time periods thereafter shown in the text. The sera were separated from the clot and stored frozen up to 4 months until tested for HI antibody by a standard procedure (3). Geometric mean titers were calculated from the individual titer values obtained from the animals given each of the vaccines.

Results. Expt. 1-537. This experiment was carried out in guinea pigs given 1 or 2 doses of aqueous or of adjuvant 65 quadrivalent influenza vaccines which were tested alone and in combination with complexed Poly I:C or Poly A:U. The animals were bled at monthly intervals up to 10 months following vaccination. The findings in the tests for an-

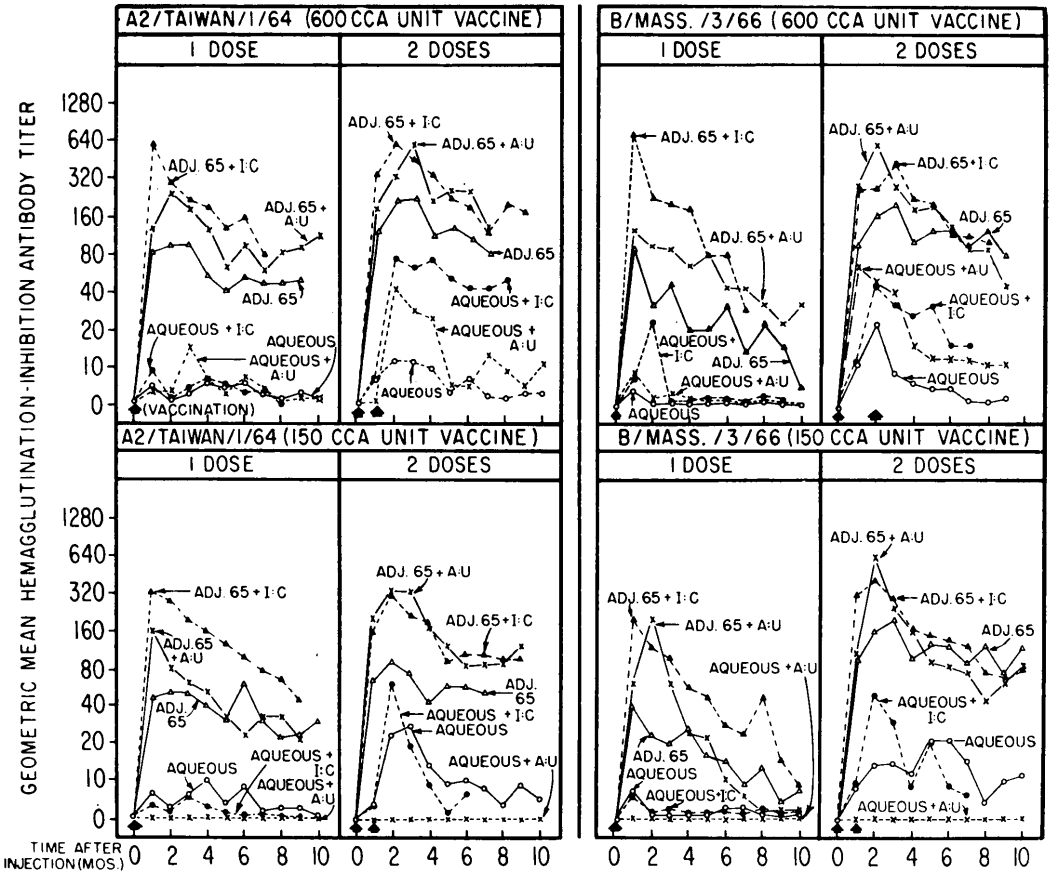

FIG. 1. Guinea pig antibody responses to polyvalent influenza virus vaccine, aqueous and adjuvant 65 types and with or without polynucleotides, given in 1 or 2 dose schedules.

tibody against A2/Taiwan/1/64 and B/Mass/3/64 viruses are shown in Fig. 1. It is seen that the homologous antibody responses to aqueous vaccine were generally poor following a single dose of vaccine but were significantly increased when 2 doses were given. The responses to aqueous vaccine were nearly always greatly improved when Poly I:C was added and were improved less frequently when Poly A:U was substituted. In some instances Poly A:U depressed the responses to aqueous vaccine. The findings for the other 2 viral antigens in the vaccine, *viz.*, A/PR8/34 and A2/Japan/170/57, were consistent with those found with A2/Taiwan and B/Mass.

The expected enhancement of antibody response to vaccine emulsified in adjuvant 65 which has been so uniformly demonstrated in the past (1, 4, 5, 8–11) was clearly demonstrated in these experiments. Remarkably, there was a striking further potentiation of antibody response when the complexed ribose polynucleotides were included in the formulations. This was far greater than the small effect which would be expected by mere addition of the polynucleotide effect obtained with aqueous antigens. Poly I:C in adjuvant 65 appeared generally to be somewhat more consistent and more active in enhancing antibody responses than was Poly A:U. The added antibody response which was obtained following use of the ribonucleotides tended to be lost after about 6 months at which time the mean titers more nearly approximated those which were obtained with adjuvant 65 vaccine alone.

Additional tests for antibody against the

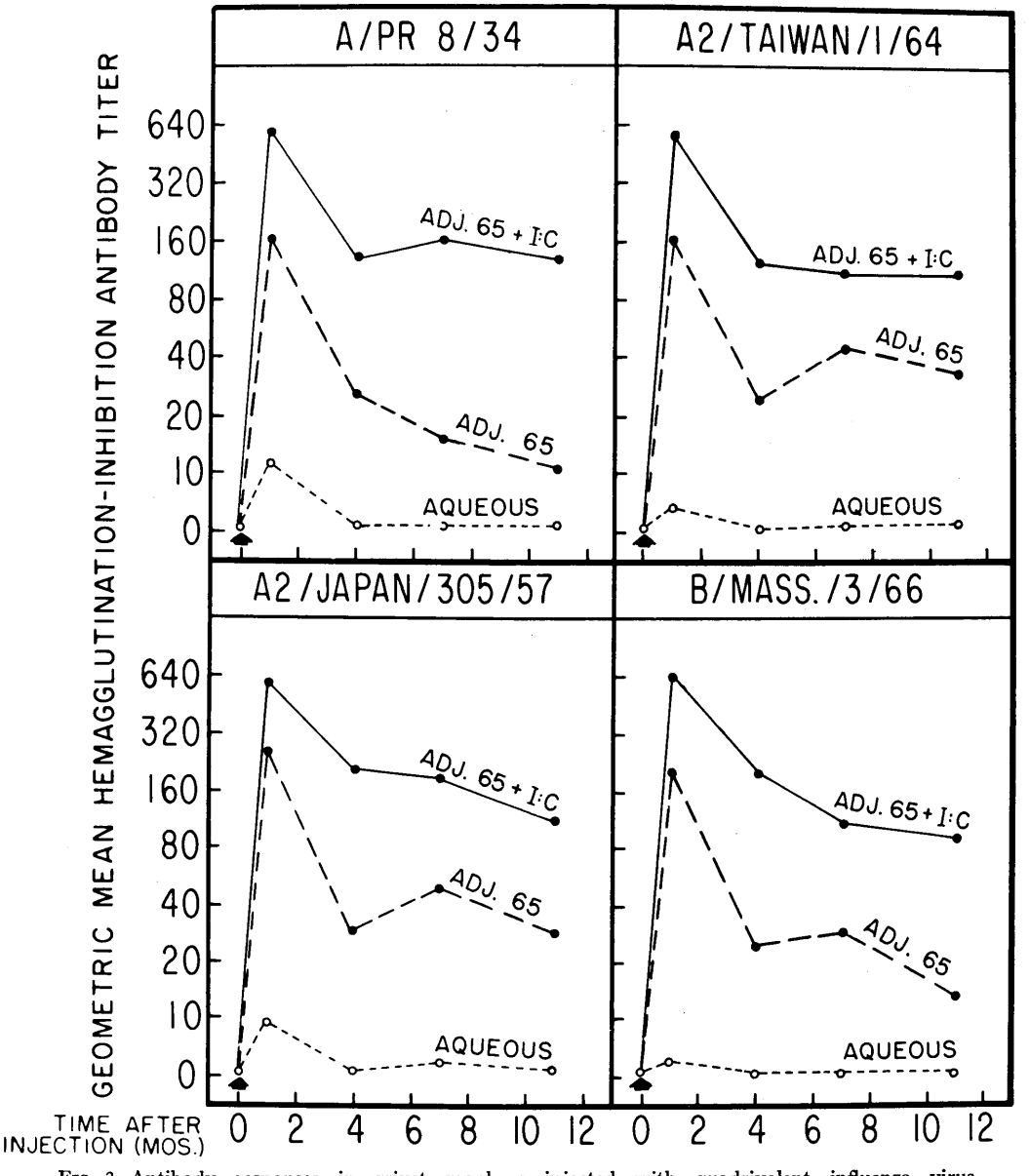

FIG. 2. Antibody responses in grivet monkeys injected with quadrivalent influenza virus vaccine in aqueous, adjuvant 65 or adjuvant 65–polynucleotide formulation.

other 2 viral antigens in the quadrivalent vaccine, *viz.*, A2/Japan/170/62 and A/PR8/34 showed essentially the same relative findings as for the 2 strains presented in Fig. 1.

Expt. 2-541. This experiment was carried out in grivet monkeys given a single dose of aqueous quadrivalent vaccine or the same vaccine in adjuvant 65 with or without added Poly I:C. The very striking enhancement of antibody response to the vaccine by use of adjuvant 65 was clearly shown (Fig. 2). The synergistic potentiation of immune response through addition of Poly I:C to the adjuvant was also clearly demonstrated. The elevation in antibody attained through use of Poly I:C was retained for at least 11 months in the monkeys, in contrast to the guinea pigs discussed above. In fact, the antibody titers

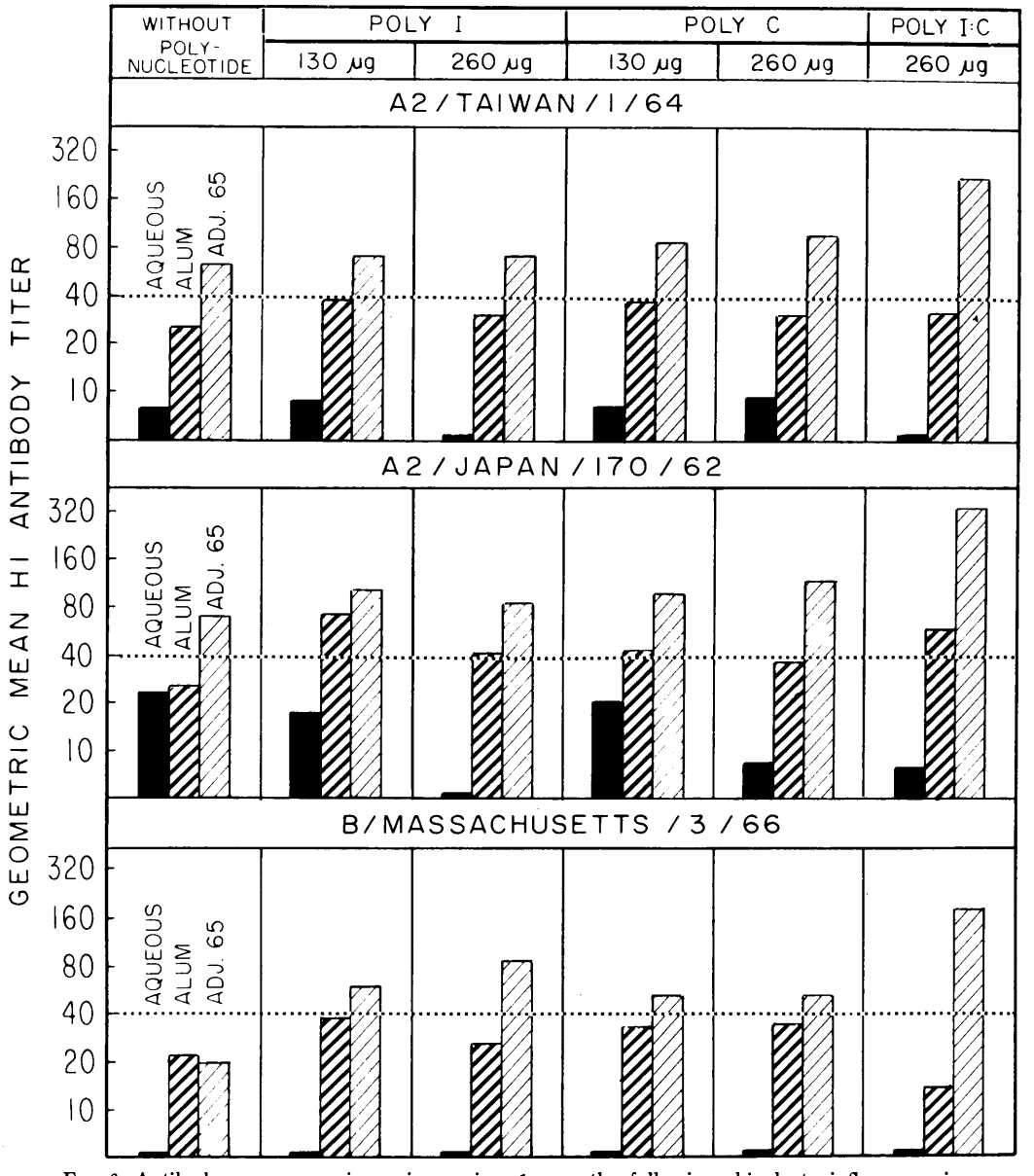

FIG. 3. Antibody responses in guinea pigs 1 month following bivalent influenza virus vaccine with or without polynucleotides and as aqueous, alum, or adjuvant 65 type formulations.

were retained significantly better following the adjuvant–Poly I:C combination than when the adjuvant was used alone.

Expt. 3-544. This experiment was carried out in guinea pigs and was undertaken for the purpose of measuring the effect of single rI and rC polynucleotides at 2 different dose levels and of the complexed Poly I:C in potentiating the antibody response to trivalent influenza vaccine in aqueous, alum, or adjuvant 65 formulation. A single dose of vaccine was given and the animals were bled 1 month later. The expected low antibody response to aqueous vaccine occurred as shown in Fig. 3. There was very marked enhancement of response to the vaccine in

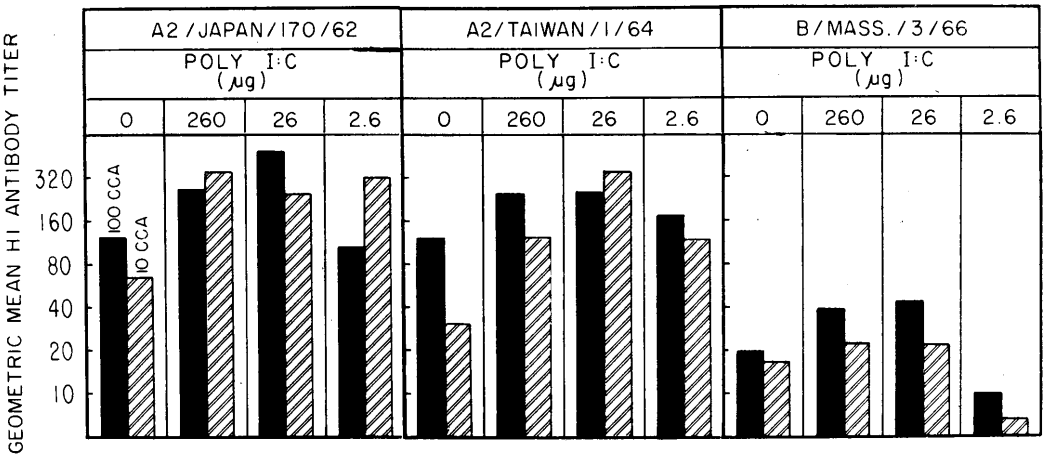

FIG. 4. Guinea pig antibody responses 1 month following injection of adjuvant 65 type bivalent influenza virus vaccine prepared at 2 antigenic concentrations and with or without different concentrations of Poly I:C.

adjuvant 65, and an intermediate potentiation of response using alum vaccine. Poly I and Poly C homopolymers alone, at either dose level, caused little if any increase in response to vaccine in any of the fomulations. There was marked potentiation when Poly I:C complex was added to the adjuvant 65 formulation. Poly I:C failed to potentiate the response to alum vaccine and, instead, might have brought about some degree of suppression.

Expt. 4-547. Guinea pigs were given monovalent influenza vaccines containing 100 or 10 CCA units of antigen in adjuvant 65 and the influence of varying doses of Poly I:C was measured. The antibody titers were measured 1 month following a single dose of vaccine. Figure 4 shows that the responses to 100-CCA unit vaccine were usually greater than to 10-CCA unit vaccine. The degree of enhancement by Poly I:C was usually greater following use of the 10-CCA unit vaccine than after the 100-CCA unit vaccine. There was little difference in the amount of enhancement of antibody response when 26 or 260 μg of polynucleotide/dose of vaccine were used. The 2.6-μg amount was insufficient with the B type influenza virus but showed some degree of enhancement with the A strains in this experiment.

Discussion. The findings in the present study confirmed two previous observations and disclosed one new phenomenon. The marked enhancement of antibody response to influenza virus antigen by adjuvant 65 (1–11) and the potentiation of serologic response to aqueous antigen by certain ribonucleotides (12) were confirmed. Newly discovered, was the demonstration of synergistic activity of adjuvant 65 combined with double-stranded ribonucleic acids (Poly I:C and Poly A:U) which effected far greater antibody levels than the additive effect of the two components tested individually.

The Poly I:C appeared to be more consistently effective in increasing the antibody responses of adjuvant 65 than was the Poly A:U. Poly A:U sometimes inhibited antibody responses to aqueous antigen; Poly I:C was rarely, if every, inhibitory. The increased antibody level in guinea pigs brought about by the addition of complexed polynucleotide to adjuvant tended to be lost by the end of about 6 months but was retained in monkeys for at least 11 months. Maximal potentiation of adjuvant 65 was obtained by 26 μg/dose of Poly I:C, 2.6 μg was insufficient with the B type of influenza virus but worked well with the A strains and 263 μg failed to increase the response beyond that obtained with 26 μg.

The mechanism of action of oil adjuvant appears to be associated (10, 11) with (a) the provision of a vehicle for transport of

emulsified antigen throughout the lymphatic system to distant sites, such as the lymph nodes and spleen where new foci of antibody formation may be established, (b) the formation and accumulation of cells of the mononuclear series which are appropriate to production of antibody at the local and distant sites, and (c) perhaps the establishment of a portion of the antigen in a persistent form at the site of injection making possible gradual release of antigen. Braun and Nakano (12) have reported that the stimulating activity of the complexed polynucleotides may be due to enhancement of the early rate of increase, in the presence of antigen, in numbers of certain antibody-forming cells and may be associated with the stimulation of nucleotide kinase activity in antibody-forming cells. The mechanism for synergistic potentiation of antibody response by use of adjuvant 65 combined with complexed polynucleotides is unknown but might be presumed to be associated with actions on antibody forming cells described above. Clearly, however, the hyperpotentiation of adjuvant activity of adjuvant 65 is not generic to all adjuvants since the addition of Poly I:C and Poly A:U to alum adjuvant was without effect.

Properly constituted aqueous influenza virus vaccines, though efficacious, are still deficient. These deficiencies relate to the short duration of immunity, lasting no more than 1 season, and to the limitation of efficacy of the vaccine often exaggerated by the perpetual antigenic alterations of the predominant virus in the population at any given time. Certain of these inadequacies have been minimized by adjuvant 65 in the stimulation of much higher level of antibody than corresponding aqueous vaccine and in the persistence of such antibody for many years. Further benefit arises from the need for smaller amounts of viral antigen to bring about the desired effect and in the remarkable broadening of antibody response to provide coverage not only against variants within a particular influenza subtype but also against markedly different viruses such as the Hong Kong influenza virus compared with the A2 strains which circulated in previous years (9). The hyperpotentiation of antibody response by the addition of complexed polynucleotides might provide a new magnitude of immunizing capability of the adjuvant for influenza and for other killed antigens. Tests to measure pathologic effects and long-term duration of antibody are currently in progress.

Summary. Immunologic adjuvant 65 caused a strong enhancement of antibody response to influenza virus antigens in tests in animals. Complexed synthetic polynucleotides Poly I:C (rI:rC) and Poly A:U (rA:rU) produced comparatively weak potentiation of antibody response to these antigens and in certain instances, Poly A:U was inhibitory. Immunologic adjuvant 65 combined with the Poly I:C or Poly A:U acted synergistically to cause a hyperpotentiation of antibody response which greatly exceeded the additive effects of adjuvant and complexed polynucleotides tested singly. Such potentiation did not occur when the complexed polynucleotides were tested with alum adjuvant or when Poly I or Poly C was added alone. Poly I:C was generally more effective than Poly A:U. Maximal potentiation was demonstrated at 26 μg/ml of Poly I:C and there was no further enhancement when the concentration was raised to 260 μg/ml. Use of adjuvant 65 combined with complexed polynucleotides promises to contribute a new magnitude of immunizing capability to be obtained when used with influenza and other killed antigen preparations.

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Alteration of ^{14}C-Dextroamphetamine Sulfate Absorption by Carbonic Anhydrase Inhibition* (33984)

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Pretreatment with acetazolamide has been shown to alter brain levels of certain drugs. Following acetazolamide treatment in rabbits Kelentey *et al.* (1) found the level of penicillin increased in brain and decreased in blood. They concluded that the increased brain levels were the result of increased permeability of the blood–brain barrier. Reed (2) found that acetazolamide treatment increased the duration of pentobarbital sleeptime in rats and the brain "pentobarbital space" was increased in treated animals during the first 20–40 min after drug administration.

Maren *et al.* (3) characterized acetazolamide as a pure carbonic anhydrase inhibitor; *i.e.*, it exerts no action either *in vivo* or *in vitro* other than the inhibition of carbonic anhydrase, the enzyme which reversibily catalyzes the interconversion of carbon dioxide and carbonic acid.

The present study was conducted to determine the effect of carbonic anhydrase inhibition on rat brain uptake of ^{14}C-dextroamphetamine sulfate, a drug producing its primary pharmacological effect in the central nervous system.

Materials and Methods. Male, Holtzman rats (140–185 g), fasted 6–12 hr, were treated with acetazolamide[1] (200 mg/kg, sc) 30 min prior to the administration of ^{14}C-*d*-amphetamine sulfate[1] (2 μCi/mg). Control animals were given an equivalent volume of saline in place of the acetazolamide. At various time intervals following the *d*-amphetamine injection, the animals were sacrificed by decapitation. Free-flowing blood was collected in tubes containing potassium oxalate. After centrifugation a 0.1-ml aliquot of plasma was taken for analysis. The brain was perfused by injecting 10 ml of saline into each carotid artery to minimize errors derived from the radioactivity in blood. Samples were taken from the frontal lobes of the cerebrum for

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