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ON THE ADJUVANT EFFECT OF ALUMINUM HYDROXIDE FOR MICE

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Linear relationships were found between the dose of A1 $(OH)_3$ adjuvant and the titer of anti-OVA antibodies formed by BDF1 mice. Mice immunized with OVA, DNP-KLH and then boosted with DNP-OVA formed anti-DNP antibodies only when A1 $(OH)_3$ was added to the injection of DNP-KLH; addition of A1 $(OH)_3$ to the priming injection of OVA decreased, rather than increased antibody formation.

Under the label of immunological adjuvants are included an array of different materials, ranging from gels or simple chemicals, such as calcium phosphate or aluminum hydroxide, to killed or live bacterial suspensions, such as *Bordetella pertussis* or BCG. (Freund, Casals & Homer, 1937); Freund (1956); Kind (1957); Dresser (1968); Hilleman & Tytell, 1971); Dresser & Philips, 1973); Mathé (1976); White (1976). Mineral gel adjuvants are the simplest of these materials, and have been extensively used in human vaccination, as well as in animal experimentation (Holt, 1949); Barr, Glenny & Butler, 1955); Vaz & Peixoto (1963); Relyveld & Raynaud (1967); Wardlaw & Aprile (1967); WHO Rech Report (1976).

The present studies were aimed at two targets. First, we examined the effect of dose of adjuvant on the formation of anti-ovalbumin antibodies in mice. Through immunization with hapten-conjugates of different carrier proteins, we then tested whether the adjuvant effect of A1 $(OH)_3$ depended on the development of T or B cells (Rajewsky et al, 1969). The results showed linear relationships between dose of A1 $(OH)_3$ and the magnitude of antibody formation, and suggested the adjuvant effect of A1 $(OH)_3$ depends on the development of B cells.

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MATERIAL AND METHODS

Mice. Female BDF1 mice, used when 7-9 weeks old, were obtained from the Jackson Laboratory, Bar Harbor, Me.

Antigens. Hens' ovalbumin (OVA, grade V, Sigma, St. Louis, Mo) and keyhole limpet hemocyanin (KLH, Pacific Biomarine Supplies, Venice, Ca), were used as native proteins or dinitrophenylated by incubation with dinitrophenyl-sulphonate (Ovary & Benacerraf, 1963); the average number of dinitrophenyl (DNP) groups per mole were respectively 9 and $84/10^4$ daltons for DNP-OVA and DNP-KLH. Bovine gamma globulin (BGG, Sigma) was used in the antibody assay as a precipitate carrier. In the assay of DNP-specific antibodies, mouse serum albumin (MSA, Sigma), dinitrophenylated as above, was used as the antigen.

Adjuvant. Al $(OH)_3$ was prepared by slow addition of 6N NaOH to an equal volume of 6N A12 $(SO4)_3$. The gel was extensively washed in water and then saline (0.15M NaCl); its final concentration was determined by dry weight.

Immunization and bleedings. Mice were immunized by intraperitoneal route. Bleedings were taken from orbital veins with Lang-Levy pipettes (H.E. Pedersen, Copenhagen, Denmark V); 0.2ml of blood was mixed with 0.9 ml of saline, the mixture allowed to clot, and the resulting serum dilution taken to represent a 1/10 dilution in the antibody assays.

Antibody assays. A modified Farr technique was use (Vaz et al, 1971) using 0.1% BGG in borate-buffered saline to increase the amount of precipitate formed in presence of 10% polyethylene glycol (Creighton, Lambert & Miescher, 1973) in substitution for amonium sulphate. For the test 0.1 ml of mouse antiserum dilutions was mixed with 01. μg of ^{125}I-OVA or ^{125}I-DNP-MSA in 0.1 ml; then 0.2 ml of polyethylene glycol was added, the tubes vortexed, incubated overnight at 4°C, centrifuged at 10,500g for 4 minutes in an Eppendorf 5411 centrifuge. The antigen binding capacity of the antisera at the 33% point (ABC-33) was determined by reference to standard curves prepared with pools of hyperimmune mouse antisera. OVA and DNP-MSA were iodinated by the chloramine-T method (Ishizaka & Okudaira, 1974).

RESULTS

Table I shows the effects of different doses of OVA and Al $(OH)_3$ on the magnitude of the antibody response. At the peak of the primary responses (day 9) only a few mice in groups receiving larger doses of OVA and Al $(OH)_3$ displayed significant responses. Four weeks later all mice received 10 μg of soluble OVA and were bled one week thereafter. This time, all animals displayed significant secondary responses. The magnitude of these responses was linearly related to the dose of A1 $(OH)_3$ as shown in Fig. 1 for the groups immunized with 0.1 and 1.0 μg of OVA.

In the next set of experiments mice were given a primary injection of either OVA or OVA plus Al $(OH)_3$; control groups were given either Al $(OH)_3$ alone or simply saline. Two weeks later the animals received an accessory immunization consisting of either 1 μg of DNP-KLH or 1 μg of DNP-KLH plus 1 mg of A1 $(OH)_3$. Two weeks thereafter, all mice were given an injection of 10 μg of DNP-OVA without A1 $(OH)_3$; they were bled one week (day 35) and two weeks (day 42) later for determination of DNP-specific antibody titers.

As shown in Table II no detectable response was present in mice which received DNP-KLH without Al $(OH)_3$, regardless of whether they had received OVA or OVA plus

TABLE I

The effect of antigen dose and adjuvant dose on antibody formation in mice

Day 0		*Day 9*	*Day 28*	*Day 35*
Al (OH)$_3$ (mg)	*OVA (μg)*	*Primary response*	*Secondary immunization*	*Secondary response*
	1.0	13 ± 3[a]	10 μg soluble OVA	285 ± 42[a]
1.0	0.3	4 ± 1	"	108 ± 38
	0.1	4 ± 0	"	140 ± 01
	1.0	7 ± 2	"	248 ± 40
0.3	0.3	4 ± 2	"	113 ± 44
	0.1	1 ± 1	"	118 ± 18
	1.0	2 ± 1	"	130 ± 34
0.1	0.3	1 + 1	"	85 ± 32
	0.1	1 ± 1	"	82 ± 39
	1.0	2 + 1	"	148 ± 18
0.03	0.3	0 + 0	"	47 ± 11
	0.1	0 ± 0	"	49 ± 36

a: ABC-33 (mean ± standard error) in groups of 4 BDF1 mice.

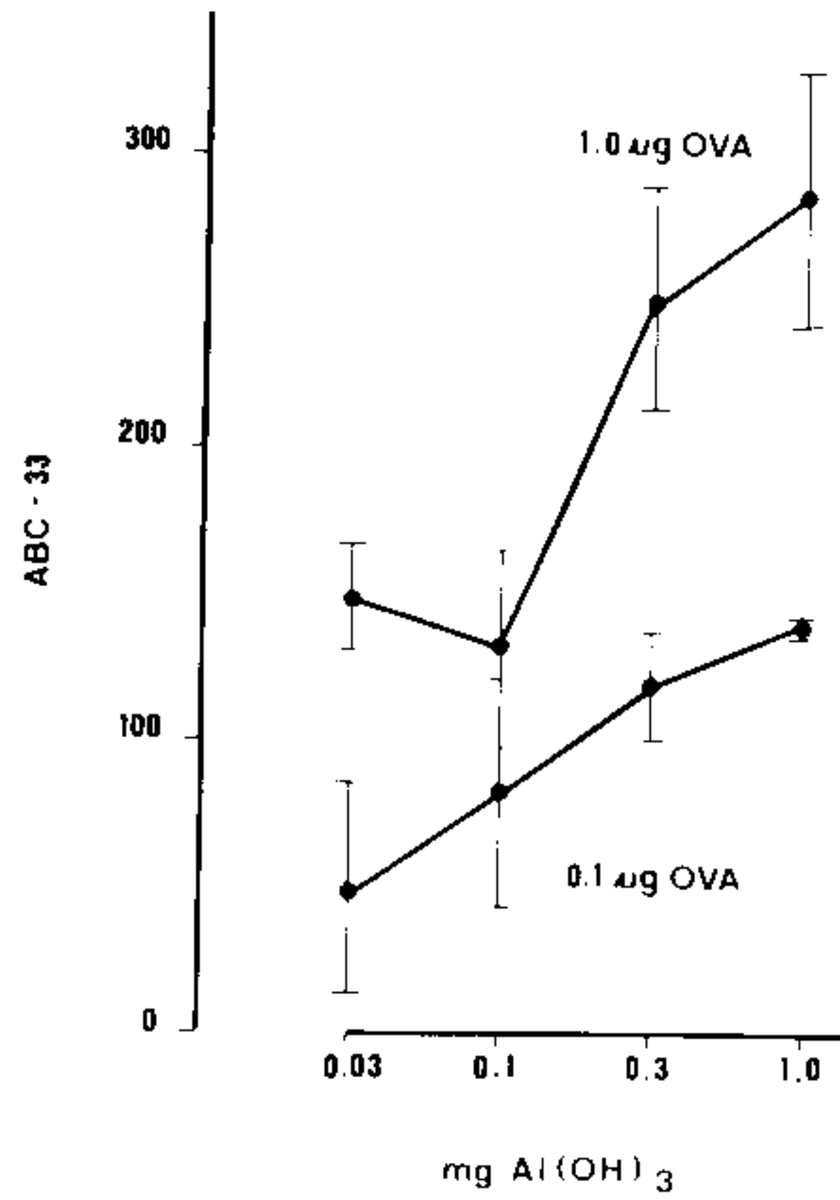

Fig. 1 – Linear relationships between log-dose of Al (OH)$_3$ adjuvant and magnitude of secondary antibody responses as measured by Farr tests in mice. In the upper curve, mice were immunized with 1.0 μg of OVA; in the lower curve, with 0.1 μg of OVA.

Al $(OH)_3$ previously. On the other hand, all animals which received DNP-KLH plus Al $(OH)_3$ displayed significant anti-DNP antibody titers; these titers were very significantly higher in mice which had received either OVA or OVA plus Al $(OH)_3$ in the primary immunization.

TABLE II

The adjuvant effect of Al $(OH)_3$ on hapten-specific secondary antibody responses

Primary[a] *immunization (Day 0)*	*Acessory*[b] *immunization (Day 14)*	*Secondary*[c] *Immunization (Day 28)*	*DNP-specific*[a] *secondary antibody titers*	
			(Day 35)	*(Day 42)*
Saline	DNP-KLH	DNP-OVA	0 ± 0	0 ± 0
Al $(OH)_3$	DNP-KLH	"	0 ± 0	0 ± 0
OVA	DNP-KLH	"	0 ± 0	1 ± 1
OVA' + Al $(OH)_3$	DNP-KLH	"	0 ± 0	0 ± 0
Saline	DNP-KLH + Al $(OH)_3$	"	4 ± 1	7 + 1
Al $(OH)_3$	DNP-KLH + Al $(OH)_3$	"	2 ± 1	8 ± 3
OVA	DNP-KLH + Al $(OH)_3$	"	19 ± 5	51 ± 14
OVA + Al $(OH)_3$	DNP-KLH + Al $(OH)_3$	"	9 ± 4	28 ± 14

a: OVA = 1 μg; Al $(OH)_3$ = 1 mg; intraperitoneal route.
b: DNP-KLH = 1 μg; Al $(OH)_3$ = 1 mg; intraperitoneal route.
c: DNP-OVA = 10 μg, soluble; intraperitoneal route.
d: ABC-33 (mean ± standard error) in groups of 5 BDF1 mice.

DISCUSSION

It has been known for quite a few years that the dose of mineral adjuvant injected with the antigen has a quantitative influence on the magnitude of immune responses (Holt, 1949); Barr, Glenny & Butler (1955); Vaz & Peixoto (1963); Relyveld & Raynaud (1967); Wardlaw & Aprile (1967). The present results allow for a detailed appreciation of this influence. The dose of Al $(OH)_3$ was, at least, as important as the dose of OVA for the induction of immune responsiveness. Thus, a tenfold reduction in the dose OVA (from 1.0 to 0.1 μg) reduced antibody titers roughly twofold; there was a similar reduction when the dose of Al $(OH)_3$ was reduced tenfold (from 1.0 to 0.1 mg or from 0.3 to 0.03 mg).

Mineral gel adjuvants such as Al $(OH)_3$, and water-in-oil emulsions, such as Freund's adjuvant (Freund, 1956) are known as depot-adjuvants, because they retard antigen elimination. But this is certainly not the only important adjuvant effect. For example: Al $(OH)_3$ or Ca_3 $(PO_4)_2$ gels cause extensive mast cell degranulation at the site of injection; there are hardly any intact mast cells in the peritoneal cavity of mice a few minutes after Al $(OH)_3$ injection. Inflammation at the site of adjuvant injection steps up the lymphatic drainage of the region (Pullinger & Florey, 1935), and thus facilitates the transport of antigen into lymph nodes. Furthermore, it is hard to exclude the presence of endotoxin in different adjuvant preparations, and endotoxin is a known polyclonal stimulator of B cells (Coutinho & Moller, 1975).

As judged from the data in Table II, B cells may be the main target of Al $(OH)_3$ adjuvant effect. The classic immunization strategy used in this experiment was first designed by Rajewsky et al (1969) and permits separate stimulation of T and B cell populations with non-cross reacting antigens; these populations are then brought together by a hapten-protein conjugate (DNP-OVA) which shares portions of the first (OVA) and second (DNP-KLH) stimulus. Table II shows that Al $(OH)_3$ was only effective when given with DNP-KLH, the B-cell generating stimulus in this schedule of immunization. Addition of Al $(OH)_3$ to the T-cell generating stimulus (OVA) decreased, rather than increased, antibody formation, an effect which might derive from the generation of suppressor T cells. Further experiments are needed to explore these possibilities.

RESUMO

Há relações lineares entre a dose de Al $(OH)_3$ usado como adjuvante imunológico e o título de anticorpos anti-ovoalbumina (anti-OVA) formado por camundongos BDFI. Camundongos que receberam OVA, depois uma imunização accessória com dinitrofenil-hemocianina (DNP-KLH), e então uma imunização secundária com DNP-OVA, só formaram anticorpos anti-DNP quando havia Al $(OH)_3$ incluído na injeção de DNP-KLH; a inclusão de Al $(OH)_3$ na injeção primária de OVA, baixou em vez de elevar o título de anticorpos anti-DNP. É sugerido que a ação adjuvante do Al $(OH)_3$ se deva a ações sobre linfócitos B.

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