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QUANTITATIVE ESTIMATION OF ANTIBODIES IN THE IMMUNOGLOBULIN CLASSES OF THE MOUSE

I. EFFECT OF ADJUVANTS ON THE ANTIBODY RESPONSE TO HUMAN SERUM ALBUMIN AND KEYHOLE LIMPET HAEMOCYANIN

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SUMMARY

Antibody activity against HSA and KLH was present in all five immunoglobulin classes of BALB/c mice. *Bordetella pertussis* vaccine was more effective in stimulating antibody production against HSA than against KLH and IgG_1 and IgG_{2b} antibody were affected more than the antibody present in the other immunoglobulin classes. The results are discussed in relation to the differing thymic dependence of these two antigens.

INTRODUCTION

Five classes of immunoglobulins have been described in mice (Fahey, Wunderlich & Mishell 1964a, b) and antibody activity has been shown to be present in all of them (Fahey *et al.*, 1964a, b; McDevitt, 1968; Warner, Val & Ovary, 1968; Wortis, Dresser & Anderson, 1969; Sell, Park & Nordin, 1970). However, no quantitative data are available on the distribution of antibody in each class and on their eventual change during the course of immunization. It has also be shown that in the mouse (Coe, 1966; Warner *et al.*, 1968) and the guinea-pig (Benacerraf *et al.*, 1963; White, Jenkins & Wilkinson, 1963) the use of different adjuvants can preferentially stimulate the production of antibodies in different immunoglobulin classes.

In an attempt to clarify some of the factors influencing the distribution of antibody in each class we have used a quantitative method to study the response of mice immunized with two protein antigens, human serum albumin (HSA) and keyhole limpet haemocyanin (KLH) in different adjuvants.

MATERIALS AND METHODS

Mice. Male BALB/c, aged between 3–4 months were used in groups of five animals.

Antigens. Human serum albumin (HSA) was obtained from Koch-Light Laboratories

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^{125}I-HSA with a specific activity >2·5 μCi/mg, was a product of the Radiochemical Centre, Amersham. Keyhole limpet haemocyanin (KLH) was purchased from Calbiochem. Co. and labelled with iodine-125 according to the method of Greenwood, Hunter & Glover (1963). *Bordetella pertussus* vaccine was obtained from Burroughs Wellcome Co., London.

Preparation of purified myeloma proteins

IgG myeloma proteins were obtained from the serum of mice bearing the RPC 23 tumour (method of Askonas, 1961) using salt precipitation and DEAE-cellulose chromatography and purified on Sephadex G-200. The Fc fragment was prepared by the method of Cebra *et al.* (1961) using insoluble papain.

IgG_{2a} myeloma proteins were obtained from C3H mice with the 5563 tumour. The myeloma protein and the derived Fc were prepared as described for the IgG_1.

IgG_{2b} myeloma proteins were obtained from BALB/c mice with the MOPC 141 tumour, purified and the Fc prepared as described for the IgG_1.

Purified IgA was obtained from the urine of BALB/c mice with the tumour 47A. The protein was precipitated with 60% saturated ammonium sulphate, the precipitate re-dissolved in water, dialysed against phosphate buffer 0·04 M, pH 8·0, and fractionated on a DEAE-cellulose column equilibrated with the same buffer. IgA was eluted with the starting buffer. For the preparation of some of the antisera the protein was further purified by isoelectric focussing using a pH gradient ranging from 4 to 6.

Purified IgM was prepared from the serum of mice with the MOPC 104 E tumour. The euglobulin fraction from these sera was fractionated on a Sephadex G-200 column and the first peak further purified on DEAE-cellulose using a gradient of molarity similar to that used for the purification of the IgG myeloma proteins.

Antisera

Antisera to mice immunoglobulins were raised in rabbits by three intramuscular injections of 1 mg of each protein in Freund's complete adjuvant given at 2-weekly intervals; the animals were bled after a further week. The resulting antisera were made specific for each immunoglobulin class by addition of myeloma protein of each of the other classes. For antisera against IgM it was necessary to use urinary Bence Jones protein type λ as the MOPC 104 E tumour used for immunization has λ type light chains while the other myelomas have κ type light chains. The absorbing antigens were added in small quantities and the resulting precipitate removed after each addition to minimize the formation of soluble complexes.

The antisera were tested for specificity first by immunoelectrophoresis and then by immunofluorescence using an acetone-fixed section of each myeloma and a goat anti-rabbit IgG labelled with fluorescein isothiocyanate. Each antiserum showed a positive reaction only when tested with the corresponding tumour. Details of the antisera are summarized in Table 1.

Estimation of the antigen binding capacity (ABC) in each immunoglobulin class

The antigen binding capacity of each immunoglobulin class was estimated as described by Torrigiani, Roitt & Doniach (1968). The principle of the method depends upon addition of an excess of radioactive antigen to the serum followed by flocculation of the complexes with a rabbit anti-immunoglobulin serum. For the estimation of antibody activity in the IgG_{2a}, IgG_{2b}, IgA and IgM fractions of mice injected with HSA, 0·025 ml aliquots of serum

were incubated in four different tubes with 100 μg of labelled antigen. After 4 hr at 37°C 0·5 ml of phosphate buffer saline pH 7·2 (PBS) was added to each tube followed by 0·2 ml of specific antiserum, a different antiserum being placed in each of the four tubes. After a further incubation at 37°C for 1 hr and at 4°C overnight, the floccules were spun down, washed twice with cold saline and counted in a Packard Auto-Gamma spectrometer, Mod. 3375. The same procedure was used for the detection of antibodies in the IgG_1 fraction except that 0·05 ml of serum diluted 1/10 was used and the amount of antigen reduced proportionally. Sera containing high levels of antibodies or antibodies to KLH were tested as for the IgG_1 but the quantity of antigen was increased to 200 μg. In each experiment two normal mouse sera were included and the highest amount of radioactivity precipitated by one of the two was subtracted from the values obtained with the sera under test.

The total ABC of the serum was obtained by summating the ABC of each of the immunoglobulin classes.

TABLE 1. Preparation of antisera

Specificity	Antigen used for immunization	Reagents used for absorption
IgG_1	Fc from RPC 23	IgG_{2a}, IgG_{2b}, IgA, IgM
IgG_{2a}	Fc from 5563	IgG_1, IgG_{2b}, IgA, IgM
IgG_{2b}	Fc from MOPC 141	IgG_1, IgG_{2a}, IgA, IgM
IgA	47A purified urinary protein	IgG_1, IgG_{2a}, IgG_{2b}, IgM
IgM	MOPC 104 E myeloma protein	IgG_1, IgG_{2a}, IgG_{2b}, IgA λ Bence-Jones proteins from mice with MOPC 104 E myeloma tumour

RESULTS

Antibody response to alum precipitated human serum albumin (HSA) and B. pertussis

Groups of mice were injected i.p. with 100 and 1,000 μg of alum precipitated HSA and 2×10^9 *B. pertussis* (AP+P). The animals were exsanguinated under anaesthesia and the antigen-combining capacity (ABC) estimated in each immunoglobulin class as described. The results are presented in Fig. 1.

In all cases no antibody activity was detected before the second week and the maximum was reached in the fourth week. There was no significant difference between the total ABC obtained with the two amounts of antigen. However, 1000 μg seemed to give rise to a moderately prolonged antibody response. Most of the activity was present in the IgG_1 class, while IgG_{2a} and IgG_{2b} accounted for only a small percentage of the total ABC of the serum. The distribution of antibody in the different IgG classes did not reflect the percentage of protein in each class: whereas the concentration of IgG_1 and IgG_{2a} was about 4 mg/ml in normal mouse serum and the IgG_{2b} was present at a concentration of about 1 mg/ml, the ABC of the IgG_{2b} class was greater than that of IgG_{2a}. As expected, the percentage of the total ABC in IgM decreased with time. In mice injected with 100 μg HSA the maximum ABC in this class was reached at 2 weeks and no antibody was detected after 4 weeks. With 1000 μg the maximum was detected at 4 weeks and a small percentage was still present at 9 weeks (Fig. 1).

IgA antibody always accounted for less than 10% of the total ABC of the serum and as with IgM the maximum was reached later and the antibody was detected for longer in mice which had received more antigen (Fig. 1). For IgM and IgA there was no significant

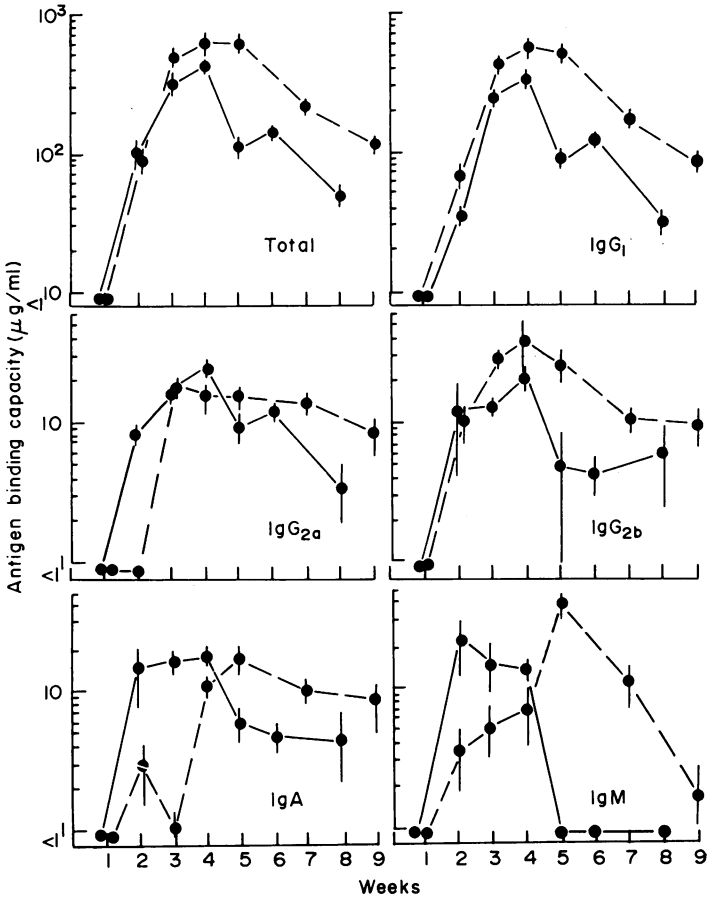

FIG. 1. Distribution of antigen-binding capacity (ABC) in the immunoglobulin classes of mice after i.p. injection of 100 μg (——) and 1000 μg (– – –) alum precipitated HSA with 2×10^9 *B. pertussis*. Values ± Standard Error.

difference between the maximum ABC found in mice injected with different amounts of antigen.

Effect of different adjuvants on the antibody response to injection of HSA

Groups of mice were injected i.p. with 1 mg. HSA given as an alum precipitate with (AP + P) or without (AP) 2×10^9 *B. pertussis* organisms or in 0·2 ml of Freund's complete (CFA) or or incomplete (IFA) adjuvant. The results obtained are reported in Fig. 2.

Mice injected with antigen in IFA produced the highest amount of antibody while those injected with AP HSA alone produced the lowest.

The maximum ABC was reached at 6 weeks in mice immunized with AP antigen while when pertussis was added the maximum was reached at 5 weeks. In mice immunized with antigen in IFA or CFA, the ABC was still increasing after 8 weeks which was the latest

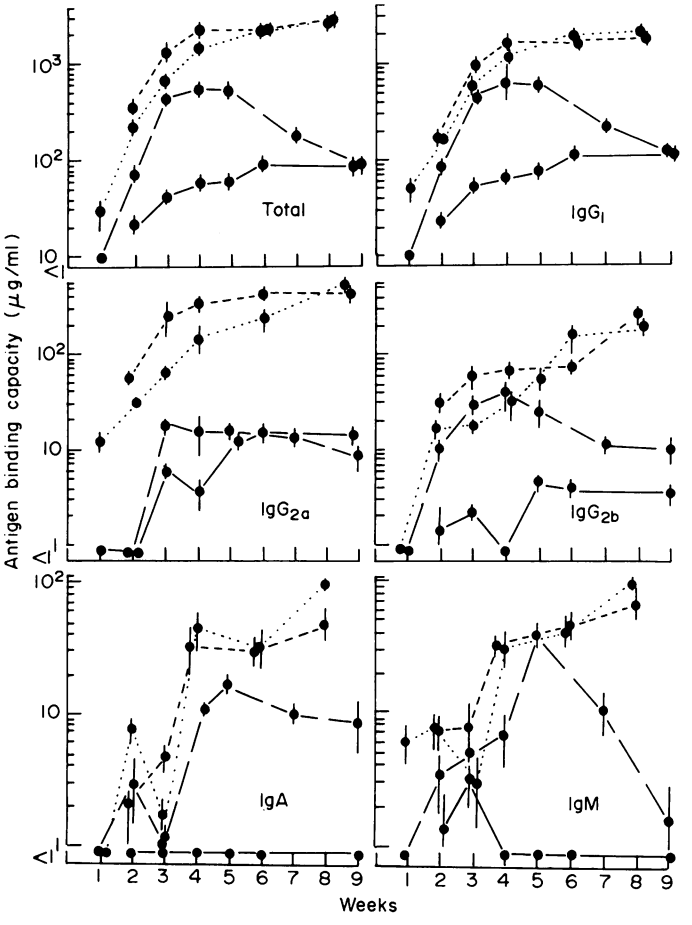

FIG 2. Distribution of ABC in the immunoglobulin classes of mice after i.p. injection of 1000 μg alum precipitated HSA with (— —) or without (——) 2×10^9 *B. pertussis* or of the same amount of antigen in incomplete (– – –) or Freund's complete (. . . .) adjuvant. Values ± Standard Error.

time tested. Addition of tubercle bacilli did not influence the maximum total ABC observed with IFA.

As in the animals immunized with AP+P, IgG_1 accounted for 80–90% of the ABC in all groups. There were, however significant differences in the relative distribution of ABC in the different IgG classes.

At two weeks the addition of *B. pertussis* to the alum precipitated antigen increased the ABC of IgG_1 3·5 times and of IgG_{2b} more than 10 times. No significant increase was found for IgG_{2a}, IgA and IgM. At 3 weeks there was a significant increase in ABC of IgG_1,

IgG_{2a} and IgG_{2b} respectively of 10, 3 and 13 times, while at 4 weeks the antibody activity in IgG_1 was increased 13 times, of IgG_{2a} 4·5 times, of IgG_{2b} 73 times and of IgM 6·5 times. At 5 weeks the ABC was significantly increased in all classes except IgG_{2a} which appeared to be relatively unaffected by pertussis. By 9 weeks only the ABC of IgA and IgM were increased. These results are summarized in Fig. 3. No antibody activity associated with IgA was detected in mice injected with antigen without *B. pertussis*.

The ratio of ABC between IgG_{2a} and IgG_{2b} was lower in animals which received *B. pertussis* (2·7–7·1) compared with those which received alum precipitated antigen alone (0·6–0·7).

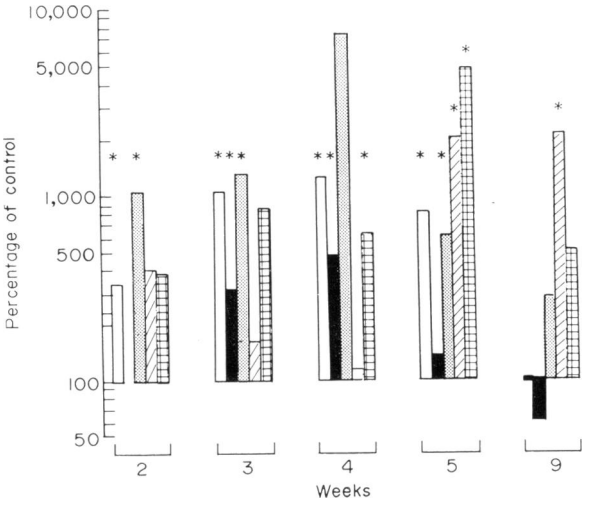

FIG. 3. Effect of *B. pertussis* on the antibody response to 1000 μg alum precipitated HSA. The results are expressed as a percentage of the control. *Difference from control statistically significant ($P<0·01$). Open colums IgG_1; Solid columns, IgG_{2a}; Stippled columns IgG_{2b}; diagonally hatched columns, IgA; Cross hatched, IgM.

In the first 4 weeks mice immunized with CFA produced less antibody than those immunized with the antigen in IFA. The reduction was more marked for IgG_{2a} and IgG_{2b} and for IgG_{2a} was still significant at 6 weeks although at this time there was no significant difference for the other classes.

There was no difference in the ratio of ABC between IgG_{2a} and IgG_{2b} between the two groups.

Antibody response to alum participated keyhole limpet haemocyanin and B. pertussis

Mice were injected i.p. with 100 or 400 μg of alum precipitated KLH plus 2×10^9 *B. pertussis*. The animals were killed at weekly intervals and the ABC estimated as described. The results are presented in Fig. 4. With both doses of antigen used the maximum ABC was reached between 3 and 4 weeks and then decreased. The difference between the two groups at a given time was not significant after the first week, although the higher amount of antigen seemed to produce a lower but slightly more prolonged antibody response. At 1 week the animals injected with 400 μg of antigen produced a significantly ($P<0·01$) higher ABC in all immunoglobulin classes. There was no obvious difference in the distribution of antibodies among the immunoglobulin classes between the groups.

Effect of different adjuvants on the antibody response to 400 μg KLH

Mice were injected i.p. with 400 μg KLH given as an alum precipitate with or without *B. pertussis* or in 0·2 ml of Freund's complete or incomplete adjuvant. The results obtained are plotted in Fig. 5.

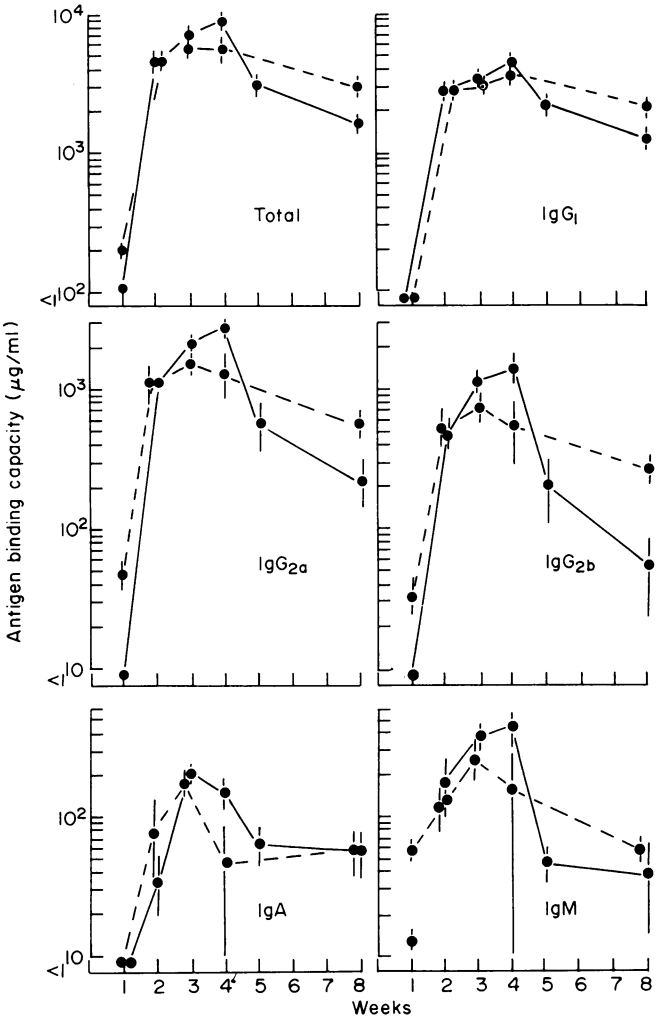

FIG. 4. Distribution of ABC in the immunoglobulin classes of mice after i.p. injection of 100 μg (——) and 400 μg (– – –) alum precipitated KLH with 2×10^9 *B. pertussis*. Values ± Standard Error.

With all the adjuvants antibodies were already present in high titre at 1 week, which was the earliest time the mice were tested. The maximum was reached at 2 weeks and remained at high levels for the duration of the experiment except in the case of mice immunized with

alum precipitated antigen which showed a rapid fall off in antibodies after the third week, in all classes. No IgA antibody was detected in these mice.

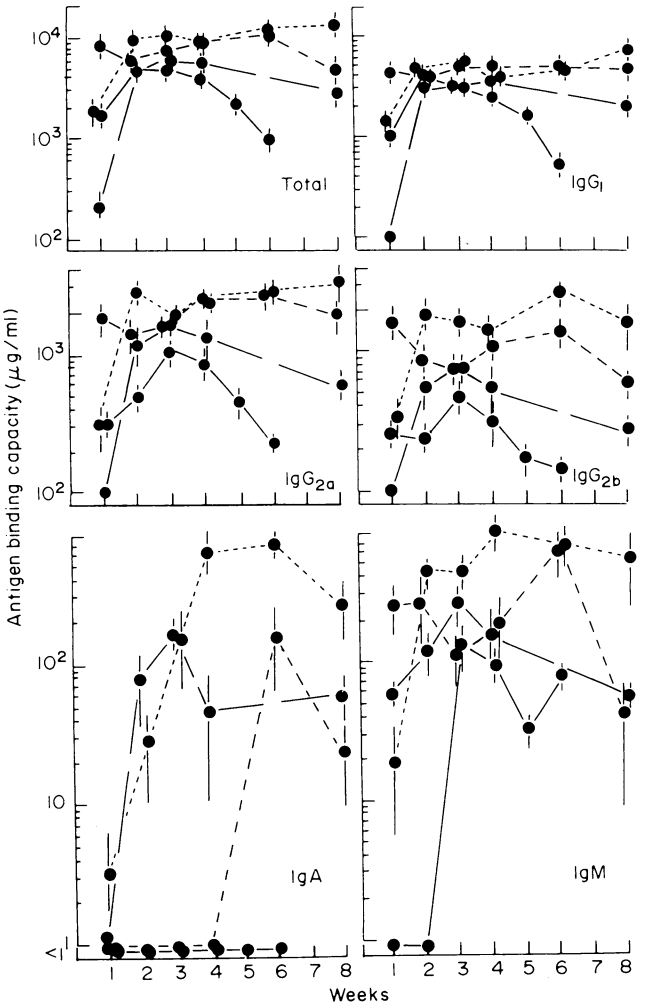

FIG. 5. Distribution of ABC in the immunoglobulin classes of mice after i.p. injection of 400 μg alum precipitated KLH with (— —) or without (——) 2×10^9 *B. pertussis* or of the same amount of antigen in Freund's incomplete (– – – –) or complete (. . . . .) adjuvant. Values ± Standard Error.

The addition of *B. pertussis* to alum precipitated KLH did not effect the antibody response in a significant way in the first 4 weeks with the exception of the first when mice injected with *B. pertussis* produced a significantly lower level of antibody in IgG_1, IgG_{2a} and IgG_2 and an increased production of IgM antibody. At 2 weeks there was a significant increase in ABC in IgM. Animals which received pertussis showed a prolonged antibody response in all classes.

At 1 week, mice immunized with CFA produced less antibody in IgG_1, IgG_{2a}, IgG_{2b} and IgM than mice which had received IFA. By the second and third weeks, however, mice immunized with CFA had a significant higher ABC in IgG_{2a} and IgG_{2b}. No difference between the two groups was found for IgG_1 or for the ratio between ABC in IgG_{2a} and IgG_{2b}.

Comparison between the percentage of ABC in the immunoglobulin classes of mice immunized with HSA and KLH

With all adjuvants a greater percentage of the total ABC was present in IgG_1 when HSA was used as antigen instead of KLH. In the latter case there was a relative higher percentage of ABC in IgG_{2a} and IgG_{2b}. A typical example is given in Fig. 6.

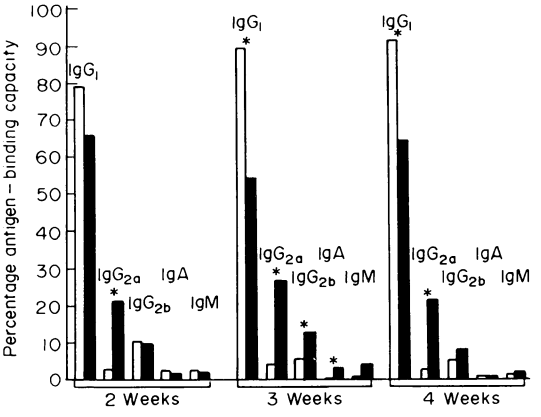

FIG. 6. Comparison between the percentage of ABC in each immunoglobulin class after injection of 1,000 μg alum precipitated HSA and 400 μg alum precipitated KLH with 2×10^9 *B. pertussis*.

* Difference statistically significant. Open columns, HSA; solid columns KLH.

DISCUSSION

Using a quantitative co-precipitation method it has been possible to show that antibody activity against HSA and KLH is present in all five immunoglobulin classes of immunized BALB/c mice.

When the antigens were injected precipitated with alum to which *B. pertussis* was added, there was no significant difference in the amount of antibody produced by the different dose of the two antigens used; however, the animals which received larger amounts of antigen gave a slightly prolonged antibody response.

It has been suggested that adjuvants increase the production of antibody mainly by three mechanisms (White, Coons & Connolly, 1955): (a) slow protracted release of antigen, (b) stimulation of macrophages, and (c) stimulation of peripheral lymphocytes. Of the adjuvants we have used, Freund's incomplete and alum may act by the first mechanism while Freund's complete and *B. pertussis* may act by all three mechanisms. HSA and KLH are known to possess different properties as antigens: at the doses used for immunization in the present study, the first is thymus dependent (i.e. the antibody response is greatly diminished by neonatal thymectomy; Humphrey, Parrot & East, 1964) and its antigenicity is greatly enhanced

by macrophage processing (Mitchison, 1969) while KLH is thymic independent (Torrigiani, in preparation) and its antigenicity is decreased by macrophages (Unanue & Askonas, 1968). Presumably in the case of a thymic independent antigen, co-operation between macrophages, T-lymphocytes and B-lymphocytes, take place only to a minor extent. The effect of the tubercle bacillus and *B. pertussis* is especially marked on thymic dependent lymphocytes as both these adjuvants favour the production of delayed hypersensitivity (Lee & Olitsky, 1955; Shaw *et al.*, 1955). Injection of pertussis causes a marked increase in circulating lymphocytes at the expense of the thymic dependent areas of the lymphoid tissue and the thymus (Taub, Krantz & Dresser, 1970). For these reasons it is not surprising that the antibody response to the thymus dependent antigen HSA is enhanced by *B. pertussis* whereas the same adjuvant has less effect on KLH. All the adjuvants have a direct stimulatory effect on the antibody forming cells that is shown by an increase in the ABC of all immunoglobulin classes against KLH. This effect is particularly noticeable for IgG_{2a} and IgG_{2b} and recalls the increase in γ_2 antibodies in guinea-pigs on incorporation of antigen in CFA (Benacerraf *et al.*, 1963; White *et al.*, 1963). The prolonged response obtained with the Freund adjuvants may be ascribed to their function as an antigen depot as suggested by White, Coons & Connolly, 1955.

In the case of HSA, *B. pertussis* stimulated IgG_1 and IgG_{2b} antibodies to a greater extent than IgG_{2a}. This effect was most clearly seen when the ratio between the ABC and IgG_{2a} and IgG_{2b} was considered. Instead of the theoretical value of 4 that would be expected from the levels of immunoglobulin in the serum, this ratio was always less than 1 in the animals treated with pertussis and always significantly lower than in the animals which received alum precipitated antigen alone. Preliminary results (Torrigiani, in preparation) indicate that the synthesis of IgG_1 antibodies is particularly thymus dependent. It is not surprising, therefore, that when a thymic dependent antigen is used, the IgG_1 class is favoured by the adjuvants which stimulate T-lymphocytes and that it also represents a higher percentage of the total antibody. IgG_{2b} is relatively unaffected by neonatal thymectomy and the stimulation by *B. pertussis* may reflect a macrophage dependence. This hypothesis is consistent with the finding that pertussis does not stimulate IgG_{2b} antibody against KLH, the antigenecity of which is decreased by macrophage processing.

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