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FACTORS INFLUENCING THE IMMUNE RESPONSE

I. EFFECTS OF THE PHYSICAL STATE OF THE ANTIGEN AND OF LYMPHORETICULAR CELL PROLIFERATION ON THE RESPONSE TO INTRAVENOUS INJECTION OF BOVINE SERUM ALBUMIN IN RABBITS

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SUMMARY

The degree of aggregation of bovine serum albumin has been shown to influence not only the quantity of antibody produced but also the magnitude and increase of the relative binding affinity of the antibodies. *Corynebacterium parvum* which causes massive lymphoreticular cell proliferation initiates 'non-specific' factors in the immune system enabling aggregate free bovine serum albumin, which normally induces unresponsiveness or hyporesponsiveness, to induce a hyperresponsive state in rabbits. This organism not only augments antibody production but the magnitude and evolution of the relative binding affinity of the antibodies.

INTRODUCTION

The 'recognition phase', 'triggering mechanisms' and synthesis of specific antibodies continue to be an enigma due to lack of a direct operational approach to study the complex mechanisms involved. *In vitro* techniques are available to study antibody synthesis but are at present limited as a means to elucidate the 'recognition phase and triggering mechanisms'. Because of the importance of the *in vivo* environment in the early stages of the immune response, it seemed desirable to study some of the factors tending to completely inhibit ('unresponsiveness'), partially suppress ('hyporesponsiveness') or augment ('hyperresponsiveness') antibody synthesis.

There are two main ways of approaching this problem. The first involving chemical and physical modification of the antigen and the second in which the cellular processes concerned in uptake and processing of antigen are altered. The physical state of an antigen can readily be altered; Dresser (1962) and Frei, Benaceraff & Thorbecke (1965) have shown that the aggregated portions of heterologous serum proteins, bovine γ-globulin (BGG) and bovine serum albumin (BSA) respectively, are highly immunogenic compared to their aggregate-free counterparts which can induce an unresponsive state. This model in which an

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antigen can act as a powerful stimulus to initiate antibody production or in a different form to induce unresponsiveness provides a means whereby the role of the cellular mechanisms of the lymphoreticular system can be studied by suitable modification of their activity.

A wide variety of substances ranging from simple chemicals to bacterial products have been shown to have effects on the lymphoreticular cells. Of these *Corynebacterium parvum* has been shown to have one of the most powerful stimulatory effects on these cells causing hepatosplenomegaly, marked increase in phagocytic activity (Halpern *et al.*, 1964) and an adjuvant effect on antibody production (Neveu, Branellec & Biozzi, 1964; Siskind & Howard, 1966; Biozzi *et al.*, 1966). In moderate doses heat-killed suspensions of *C. parvum* are not toxic to newborn rabbits and in our hands fewer neonatal deaths and an increased weight gain was found in treated compared to untreated animals (Pinckard & Weir, unpublished results). This material therefore in combining powerful stimulatory effects on the lymphoreticular tissues with lack of toxic side effects appeared to be eminently suitable for our purposes.

It is now well established that most of the classical serological tests, which measure the secondary effects of antigen–antibody interaction, select or are more sensitive towards certain populations of antibody depending on the antigen being studied. The present study was designed to assess the entire humoral immune response by measuring the antigen-binding capacity (ABC) as determined by the ammonium sulphate method of Farr (1958) and for this reason BSA was used as the antigen in this and subsequent studies. This technique enables the estimation not only of quantitative differences in antibody production but also changes in the relative binding affinity of the antibodies produced.

MATERIALS AND METHODS

Animals

Three-month-old, male and female, New Zealand White rabbits weighing from 2·0 to 2·5 kg were employed.

Antigens

Bovine serum albumin (Cohn Fraction V, Armour Pharmaceutical Lot KHO270) was used throughout this study.

Native bovine albumin (*NBA*). A 100 mg/ml solution of NBA was prepared by carefully dissolving 10 g of bovine serum albumin in 0·85% NaCl and bringing the final volume to 100 ml; the NBA solution was then sterilized by Seitz filtration and stored in aliquots at −20°C.

Centrifuged bovine albumin (*CBA*). Four millilitre quantities of NBA were centrifuged at 40 000 rev/min for 60 min ($8{\cdot}2 \times 10^6$ g-min) in an MSE Superspeed 40, 3×5 ml swing-out rotor. At the end of the 60-min centrifugation period the rotor was allowed to come down without braking and the top 2 ml of solution of each tube was carefully drawn off and used immediately.

Alum-precipitated centrifuged bovine albumin (*ACBA*). ACBA was prepared as the aluminium hydroxide precipitate by adding with mixing 50 mg of CBA to a mixture of 6 ml of Alhydrogel (1·3% Al_2O_3) and 6 ml of sterile 0·85% NaCl. The mixture was allowed to equilibrate at room temperature for 1 hr and then overnight at 4°C. The precipitate was

centrifuged at 1000 ***g*** for 10 min and the supernatant discarded. The precipitate was then suspended in 2–3 ml of sterile 0·85% NaCl and passed through a 23 gauge needle 3–4 times to break up any large aggregates. This ACBA precipitate has been found to be a highly stable preparation and does not appreciably dissociate even when incubated in the presence of 1/10 normal rabbit serum at 37°C for 1 week (Pinckard & Weir, unpublished results).

Preparation of Corynebacterium parvum

Cultivation media. C. parvum strain 10387 was originally obtained from the National Collection of Type Cultures, Colindale, London. The organism was grown anaerobically in a special cooked-meat broth containing 1% glucose. To ensure that the cooked-meat broth was devoid of bovine antigens, it was prepared from sheep muscle as follows: 500 g of sheep heart, trimmed free of all fat and connective tissue, was finely minced and boiled for 20 min with 500 ml of distilled water and 1·5 ml of 1 N-NaOH. The resulting suspension was filtered through muslin and the solid material was blotted dry. The minced cooked heart was then distributed in 100-ml and 500-ml bottles to a depth of 1–2 in. and this was overlaid with a sheep meat infusion broth until each bottle was 4/5th full. The infusion broth was prepared from 500 g of lean sheep meat which was minced and mixed with 1 litre of distilled water containing 5 g NaCl. This mixture was held for 24 hr at 4°C. It was then boiled for 15 min, filtered through muslin and then filter paper. The pH of the filtrate was then adjusted to 7·4 with 1 N-NaOH. After the bottles were filled with the desired amount of minced cooked heart and infusion broth, they were sealed and autoclaved at 121°C at 15 lb pressure for 15 min.

Cultivation of C. parvum. Just prior to inoculation with the stock culture, each bottle of culture medium was enriched with sufficient Seitz-filtered sterile 10% glucose solution to bring the glucose concentration to 1%. The bottle was then sealed loosely with its screw cap and steamed for 20 min to ensure anaerobic conditions. A 100-ml bottle of the cooked-meat broth described above was inoculated with *C. parvum* and incubated anaerobically at 37°C for 48 hr. The growth of this starter culture was checked for purity by examination of Gram-stained smears and by subculture on blood agar enriched with glucose. Meanwhile, a 25-ml volume of the starter culture was inoculated into each of four 500-ml bottles of culture medium and these were incubated (without shaking) for 3 days at 37°C under anaerobic conditions.

Preparation of C. parvum for injection. The *C. parvum* cultures were filtered through eight layers of muslin in order to remove any meat particles. The resulting suspension was centrifuged at 2000 ***g*** for 30 min and the supernate discarded. The bacterial deposits were resuspended in 0·85% NaCl and washed 6 times. After the last centrifugation, the pellet was resuspended in 3 volumes of 0·85% NaCl. The final suspension of *C. parvum* was then heat-killed by incubation in a 70°C water bath for 60 min. The suspension was then finally checked for sterility and if the suspension was not immediately used enough formalin was added to make a final concentration of 1%. The dry weight of the suspension was then determined by heating 1·00 ml of the suspension in a tared flask at 100°C until constant weight was established.

Measurement of the antigen-binding capacity

The antigen-binding capacity (ABC) was determined by the ammonium sulphate method described by Farr (Farr, 1958; Minden & Farr, 1967). All sera were diluted 1:10 and then

three-, four- or five-fold dilutions were made depending upon the stage of the immune response from which the serum was taken and also the anticipated ABC .The end point was that serum dilution which bound 33% of the antigen and the ABC's were expressed as μg N BSA bound/ml of undiluted sera. Parallel tests were run on each serum employing two concentrations of traced labelled [^{131}I]BSA (0·02 μg N BSA and 0·20 μg N BSA). The effect of dilution of the antigen upon the antigen-binding capacity was determined as (ABC 0·02 μg N BSA/ABC 0·20 μg N BSA) × 100 and gives an indication of the relative binding affinity of the antibodies present in the serum. A standard serum was employed in order to ensure accuracy in the ABC determinations from run to run.

Preparation of trace labelled [^{131}*I*]*BSA* (*BSA**)

The Chloramine-T method of Hunter & Greenwood (1962) was employed. In a small reaction flask containing 2–3 mCi carrier-free iodine-131, 0·2 ml of borate buffer, pH 8·4, was added. Five milligrams of BSA in 0·5 ml borate buffer was then added with rapid stirring followed immediately by 100 μg of Chloramine-T in 0·2 ml distilled water. The solution was allowed to react for 60 sec and then 4 ml of borate buffer was added. The solution was dialysed against borate buffer for 24 hr and then against numerous changes of borate buffer containing potassium iodide carrier for three additional days. The concentration of BSA* in μg N/ml was determined by the Micro-Kjeldahl method (Kabat & Mayer, 1961). The BSA* preparations were used only if greater than 99% of the radioactivity was precipitable by 10% trichloroacetic acid. The efficiency of ^{131}I incorporation by this method in this laboratory has been greater than 90%. All counting measurements were performed in a Nuclear Enterprises Gammamatic well-type scintillation spectrometer with a 2-in. sodium iodide crystal.

Immunization and bleeding schedules

All of the injections were made into the marginal ear vein. Each rabbit receiving *C. parvum* was injected with 10 mg of the heat-killed suspension of *C. parvum* 6 days prior to the injection of 50 mg BSA antigen. The rabbits were bled every 3rd day after the injection of BSA up to day 21 and then again at day 30 just prior to the secondary injection of 50 mg BSA. All sera were stored at −20°C.

RESULTS

Primary response

Six parallel experiments were run, three groups receiving intravenous injections of 50 mg of NBA, CBA and ACBA respectively on day 0. The three remaining groups were injected with 10 mg of a heat-killed suspension of *Corynebacterium parvum* at −6 days and then intravenous injections of 50 mg of NBA, CBA or ACBA respectively on day 0. The results of these experiments are seen in Fig. 1 which reflects the level of anti-BSA antibodies produced and in Fig. 2 which shows the increase in relative binding affinity of the antibodies.

Antigen-binding capacity. The results expressed in Fig. 1 show that the antigen-binding capacities of those rabbits in the control group receiving ACBA responded to a greater degree than either the CBA or NBA injected rabbits. In contrast all three groups receiving *C. parvum* gave similar ABC values; however, it can be seen that there is a marked difference

between the control CBA and the *C. parvum*–CBA groups both in ABC levels and the number of rabbits responding. This situation is similar in the control NBA and *C. parvum*–NBA groups with the exception of rabbit B37. There was no significant difference however between the control ACBA and *C. parvum*–ACBA groups. It should be noted in the control

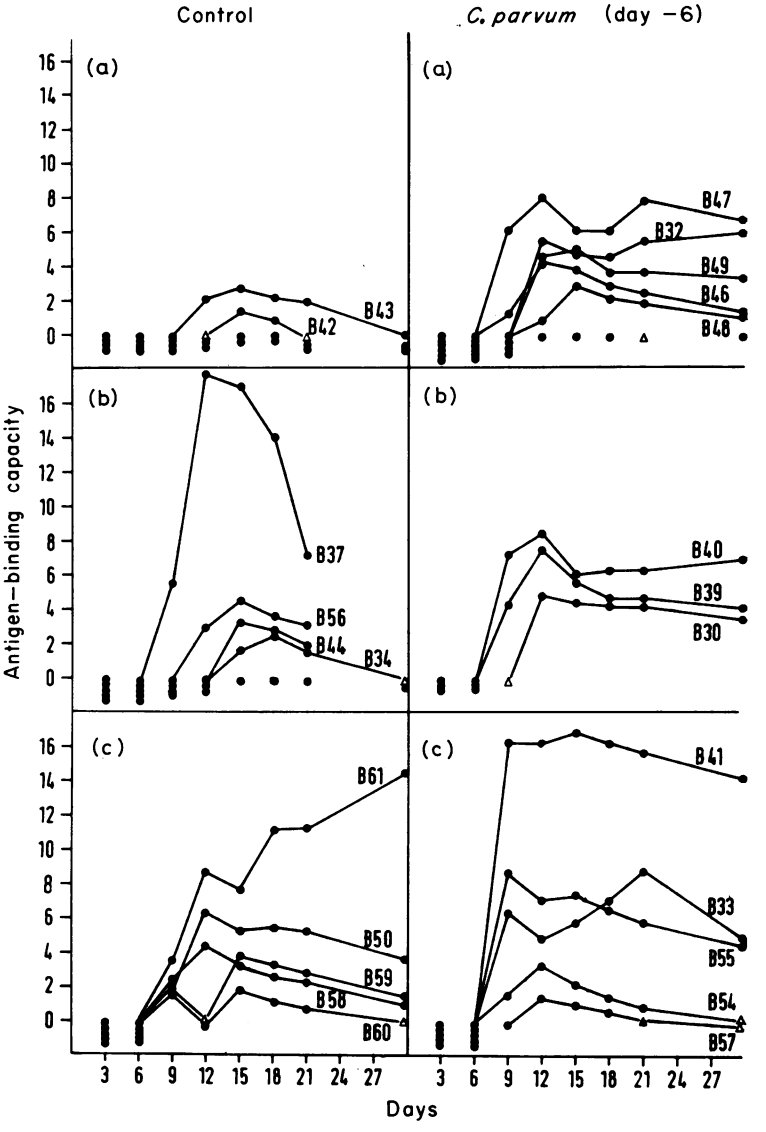

FIG. 1. Primary response: antigen-binding capacity (Reciprocal ABC-33 end point $\times 0{\cdot}20$ μg N BSA $\times$ $2 \times 0{\cdot}33$) of control and *Corynebacterium parvum* (−6 days) groups given intravenous injections of: (a) centrifuged bovine albumin (CBA), (b) native bovine albumin (NBA), or (c) alum-precipitated centrifuged bovine albumin (ACBA) on day 0. △, Insufficient antibody to give a positive ABC at a 1:10 dilution.

ACBA and *C. parvum*-treated groups, that a large number of the rabbits elicited a 'biphasic' type and sustained immune response with respect to ABC magnitude.

Effect of dilution. Fig. 2 demonstrates the effect that dilution of the antigen has upon the ABC values which, as stated previously, gives an indication of the relative binding affinity

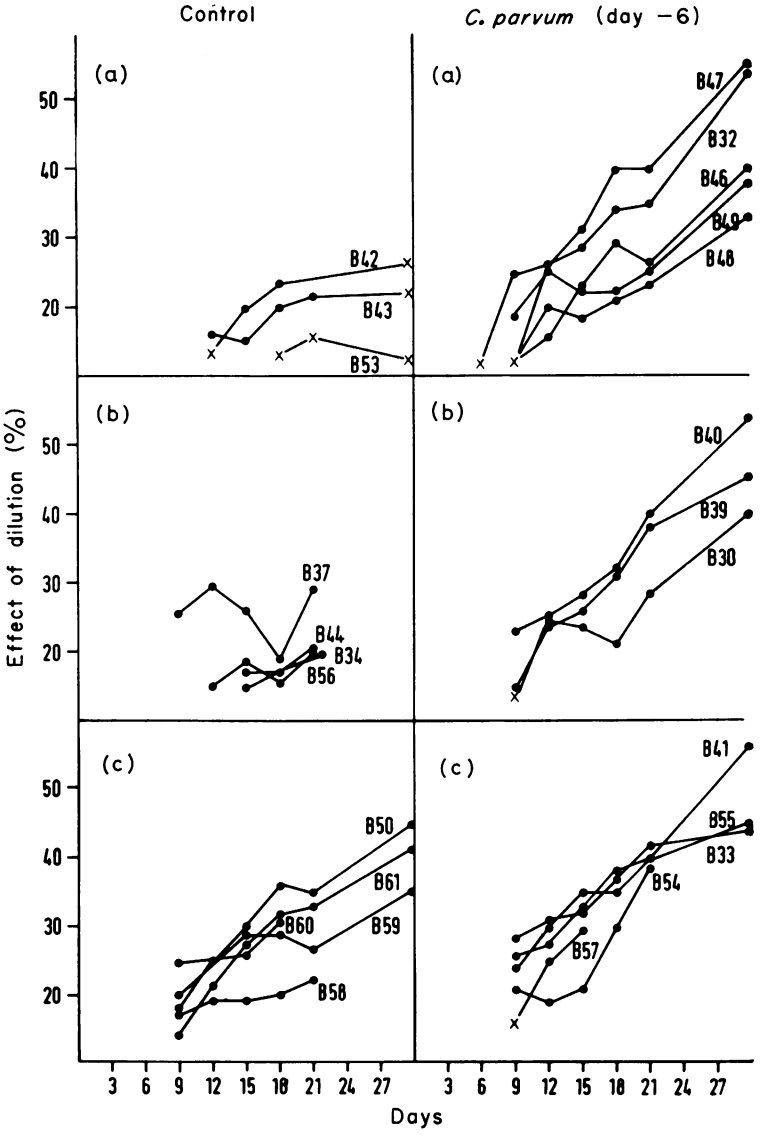

FIG. 2. Primary response: effect of dilution % [(ABC-33, 0·02 μg N BSA/ABC-33, 0·20 μg N BSA) × 100] of control and *Corynebacterium parvum* (−6 days) groups given intravenous injections of: (a) CBA, (b) NBA or (c) ACBA on day 0. ×, Estimated effect of dilution % from % binding of a 1:10 dilution of antiserum.

of an antiserum; this enables qualitative comparisons to be made of the antisera produced by the various groups. It is apparent that not only are the values of the effect of dilution % greater in control ACBA and *C. parvum*-treated groups than in the other groups, but the slopes of increase are steeper in these groups. This indicates that not only is there more

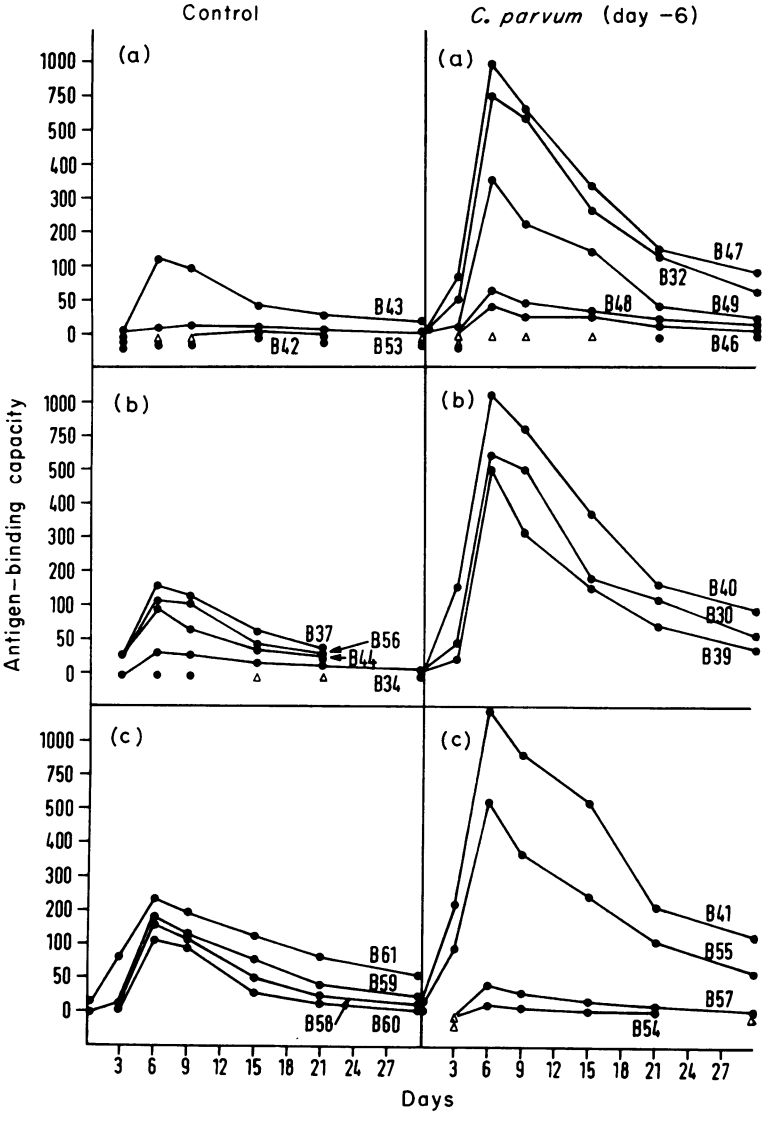

FIG. 3. Secondary response: antigen-binding capacity (Reciprocal ABC-33 end point ×0·20 μg N BSA ×2×0·33) of control and *Corynebacterium parvum* (−6 days) groups given secondary injections of: (a) CBA, (b) NBA or (c) ACBA on day 30 of the primary response (i.e. day 0 of the secondary response). △, Insufficient antibody to give a positive ABC at a 1:10 dilution.

antibody produced in control ACBA, and *C. parvum*-treated groups as seen in Fig. 1, but the antibodies produced have a greater affinity for the BSA molecule.

Secondary response

At day 30 of the primary response, all of the rabbits were given intravenous injections of 50 mg of the bovine albumin antigen in the same form as they received for the primary

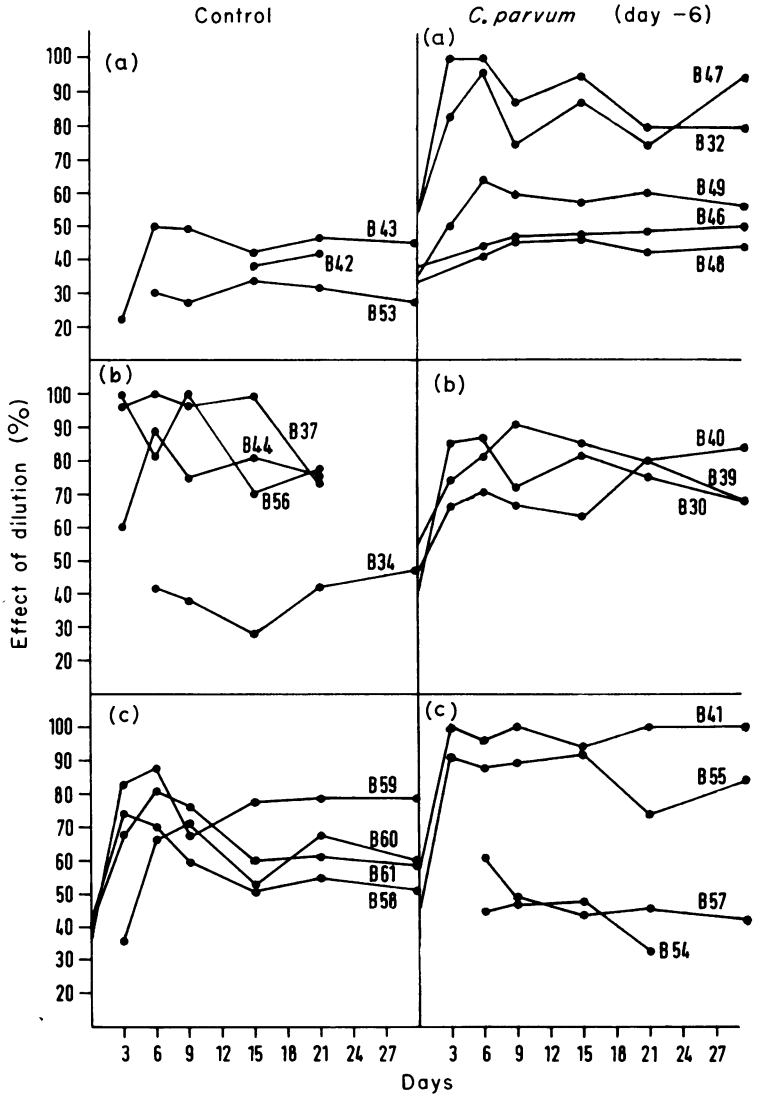

FIG. 4. Secondary response: effect of dilution % [(ABC-33, 0·02 μg N BSA/ABC-33, 0·20 μg N BSA) × 100] of control and *Corynebacterium parvum* (−6 days) groups given secondary injections of: (a) CBA, (b) NBA or (c) ACBA on day 30 of the primary response (i.e. day 0 of the secondary response).

stimulus. The groups receiving *C. parvum* before the primary stimulation did not receive a further dose of the organism before the secondary stimulation.

Antigen-binding capacity. The results of the quantitative production of anti-BSA antibodies are seen in Fig. 3. It is evident that a greater percentage of rabbits receiving *C. parvum* prior to primary stimulation with bovine albumin antigens, irrespective of their physical state, become hyperreactive shortly after secondary exposure to the antigen.

Effect of dilution. In all of the groups (Fig. 4) there is in general an immediate, rapid increase in the effect of dilution % which then levels off into a plateau. It is noteworthy in the *C. parvum*–CBA group that there is a significant difference in the magnitude of the relative binding affinity as compared to the CBA control group.

DISCUSSION

The results in the control groups of animals given BSA in different physical states substantially confirms the results of the earlier work of Dresser (1962) and Frei *et al.* (1965) in which centrifuged or *in vivo* filtered antigen results in unresponsive or hyporesponsive states. We have in addition shown that the physical state of the antigen influences the relative binding affinity of the antibodies produced. The knowledge that aggregated and/or denatured proteins are more avidly phagocytozed by the reticulo-endothelial system (Thorbecke, Maurer & Benacerraf, 1960) and the apparent dependence of the immune response on phagocytosis (Fishman, van Rood & Adler, 1965; Mowbray & Scholand, 1966) suggests at least a two-stage mechanism for the synthesis of antibody, the first involving antigen capture and processing and the second specific stimulation and proliferation of the antibody producing cells. Furthermore, it has been proposed that a 'non-specific' stimulatory mechanism acting upon lymphoid elements in conjunction with specific antigen stimulation is necessary to potentiate antibody production (Claman, 1963; Talmage & Pearlman, 1963).

Corynebacterium parvum has been shown to induce considerably enhanced phagocytic activity of the reticulo-endothelial system and massive hyperplasia of lymphoid tissue (Halpern *et al.*, 1964). Injection of only 255 μg of *C. parvum* into mice causes in 8 days an increase in the phagocytic index of 770%. *C. parvum* induces in the liver a two-fold weight gain and histologically an increase in numbers of Kupffer cells and numerous nodules localized in the portal spaces caused by infiltration of histiocytic elements. In the spleen there is a three-fold weight gain, extensive hyperplasia of the red and white pulp, increase in numbers of histiocytes and lymphocytes in the lymphoid folicles and an increase in the number of giant cells (Halpern *et al.*, 1964). In view of these powerful effects on the cells of the lymphoreticular system it was considered that *C. parvum* would be expected to potentiate antibody production by acting as a 'non-specific' stimulator.

It is clear from our results that *C. parvum* has a profound effect upon antibody production to BSA in rabbits. The observed augmentation is not only reflected in the quantity of anti-BSA antibodies produced but in the magnitude and more rapid evolution of the relative binding affinity of the antibodies. It has been shown that *C. parvum* when incorporated into incomplete Freund's adjuvant produces delayed hypersensitivity and increased antibody production to picrylated proteins in guinea-pigs (Neveu *et al.*, 1964). Siskind & Howard (1966) have demonstrated that *C. parvum* when injected shortly before the injection of SII pneumococcal polysaccharide augments the production of immunity in mice to subsequent

challenge by live pneumococcal organisms as measured by survival rate. Recently Biozzi *et al.* (1966) have shown that *C. parvum* not only produced an increase in the number of immunologically active spleen cells against erythrocytes, but also indicated that the amount of antibody produced by each cell was increased. This latter finding could also be explained by *C. parvum* inducing the formation of higher affinity antibodies, as indicated by our observations, which might well result in higher agglutination titres. The induction of delayed hypersensitivity by Neveu *et al.* (1964) could also be explained in the same way in view of the possible role of high affinity antibodies in delayed hypersensitivity as postulated by Karush & Eisen (1962).

The differences observed between the control CBA and *C. parvum*–CBA groups are noteworthy. When CBA is injected into 'normal' rabbits a high percentage of the animals are rendered hyporesponsive or unresponsive, and rabbits previously injected with *C. parvum* and then CBA become hyperresponsive to this antigen to a level greater than even when ACBA is injected into 'normal' rabbits The term 'normal' is used since it would be anticipated that rabbits continually experience subclinical infections with Gram-negative bacteria and coccidial organisms; these organisms and other environmental stimuli are known to possess adjuvant activity (Munoz, 1964) and might in part explain why in the present study or that of Frei *et al.* (1965) some of the rabbits given CBA respond; genetic influences on immunity in rabbits and mice to BSA must also be important here (Sobey, Magrath & Reisner, 1966). The two points relevant to the CBA injected groups which need comment and further study are, firstly, what are the mechanism(s) involved in CBA induced unresponsiveness or hyporesponsiveness and at what point does *C. parvum* 'non-specifically' effect these mechanism(s) and, secondly, how and why does *C. parvum* augment not only the quantity of antibody produced but the relative binding affinity of these antibodies?

The answers to these questions may help to elucidate the cellular mechanisms leading to antibody formation particularly if the different effects which *C. parvum* has on the lymphoreticular cells can be separated one from the other.

Whether this phenomenon of non-specific stimulation occurs during the course of infective disease in humans is a matter of speculation; also one can not rule out the possibility that immunological anomalies such as allergic and autoimmune disorders may be partially potentiated by 'non-specific' factors due to infective processes and environmental stimuli.

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