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FACTORS INFLUENCING THE IMMUNE RESPONSE

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

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SUMMARY

The injection of *Corynebacterium parvum* at the same time as centrifuged bovine albumin has been shown not to have the adjuvant effect found when *C. parvum* is injected 6 days before. The implication of this is discussed and related to mechanisms of antibody synthesis.

Whereas particulate alum-precipitated centrifuged bovine albumin was shown to be more effective than centrifuged bovine albumin in inducing primary antibody stimulation, the reverse was true for secondary stimulation by the intraperitoneal route.

INTRODUCTION

It has been shown in a previous communication (Pinckard, Weir & M^{c}Bride, 1967) that the degree of aggregation of bovine serum albumin (BSA) and/or changes in lymphoreticular cells induced by *Corynebacterium parvum* influence not only the quantities of anti-BSA antibodies produced but also the quality of these antibodies with respect to their relative binding affinity. Recently Neveu, Branellec & Biozzi (1964), Siskind & Howard (1966) and Biozzi *et al.* (1966) have also demonstrated adjuvant effects of *C. parvum* to various other antigens. Further to the work described in the preceding paper in which the intravenous route was used for immunization, it was decided to test the effects of the physical state of the antigen and lymphoreticular cell proliferation induced by *C. parvum* on the antibody response to intraperitoneal injection of BSA; it would be anticipated that the distribution of antigen in the lymphoid tissue would be different from that when the intravenous route is used. It is known that the intraperitoneal route is inferior to the intravenous with respect to antibody ormation to soluble protein antigens; Dresser (1962) used this route to induce unresponsiveness in mice to bovine γ-globulin. The importance of the route of administration of antigen was shown by Battisto & Miller (1962) who demonstrated

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that adult guinea-pigs could be more easily rendered unresponsive to hapten–protein conjugates if the antigen was injected into the mesenteric vein.

This second study was also modified to determine whether the time relationships of *C. parvum* administration and antigen injection are important; in particular to show whether *C. parvum* given simultaneously with BSA antigens would produce the same adjuvant effects as *C. parvum* injected 6 days prior to the injection of the antigen. If the action of *C. parvum* is dependent upon proliferation of lymphoreticular cells thereby promoting antigen capture and proliferation of specifically-stimulated cells, then it would be anticipated that rabbits injected at day 0 with both *C. parvum* and BSA would elicit a similar type of immune response but would be delayed by 6 days as compared to rabbits receiving *C. parvum* at day −6 and then BSA at day 0. If on the other hand the rabbits given *C. parvum* simultaneously with BSA do not give an augmented immune response or become unresponsive when given centrifuged bovine albumin, then *C. parvum* would appear to initiate 'non-specific' factors which either directly interfere with the induction of unresponsiveness or accelerate the 'recognition phase' and/or 'triggering mechanism' thereby priming the immune system before the unaggregated protein can induce unresponsiveness.

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 KH0270) was used. Centrifuged bovine albumin (CBA) and alum-precipitated centrifuged bovine albumin (ACBA) were prepared by the methods presented in the preceding paper (Pinckard *et al.*, 1967).

Preparation of Corynebacterium parvum

The *C. parvum* was prepared as previously described (Pinckard *et al.*, 1967).

Measurement of the antigen-binding capacity

The antigen-binding capacity was determined by the ammonium sulphate method of Farr (Farr, 1958; Minden & Farr, 1967) and the Chloramine-T method of Hunter & Greenwood (1962) was employed for preparing the [^{131}I]BSA, as described in the preceding paper (Pinckard *et al.*, 1967).

Immunization and bleeding schedules

All the rabbits were injected into the marginal ear vein with 15 mg of *C. parvum* either on day −6 or day 0. On day 0 all rabbits were injected intraperitoneally with 50 mg of either CBA or ACBA. The rabbits were bled every 3rd day after the injection of BSA up to day 18 and then again at day 30 just prior to the injection of 10 mg of the respective BSA antigen. All sera were stored at −20°C.

RESULTS

Primary response

Four parallel experiments were run, two groups injected with 15 mg of *Corynebacterium parvum* into the marginal ear vein on day −6 and then injected intraperitoneally with 50 mg of either CBA or 50 mg ACBA. The two remaining groups were simultaneously injected with 15 mg of *C. parvum* into the marginal ear vein and either 50 mg of CBA or 50 mg of ACBA intraperitoneally. The results of these experiments are seen in Fig. 1 which reflects the level

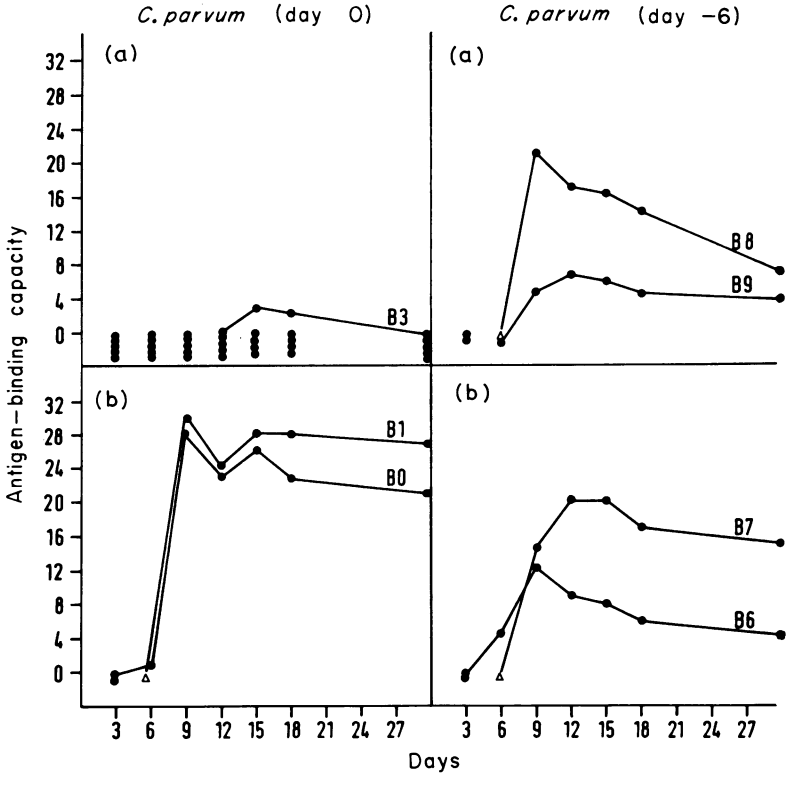

FIG. 1. Primary response: antigen-binding capacity (Reciprocal ABC-33 end point ×0·20 μg N BSA × 2 × 0·33) of *Corynebacterium parvum* (day 0) and *C. parvum* (−6 days) groups given intraperitoneal injections of: (a) centrifuged bovine albumin (CBA), or (b) alum-precipitated centrifuged bovine albumin (ACBA) on day 0. △, Insufficient antibody to give a positive ABC at a 1:10 dilution.

of anti-BSA antibodies produced and in Fig. 2 which show the increase in relative binding affinity of the antibodies.

Antigen-binding capacity. The results given in Fig. 1 show that rabbits either given *C. parvum* and ACBA on day 0 or rabbits given *C. parvum* on day −6 and ACBA or CBA on day 0

elicit a strong primary antibody response as compared with rabbits given *C. parvum* and CBA on day 0.

Effect of dilution. In all of the groups with the exception of the rabbits receiving *C. parvum* and CBA on day 0, there is a constant, rapid increase in the effect of dilution % reflecting

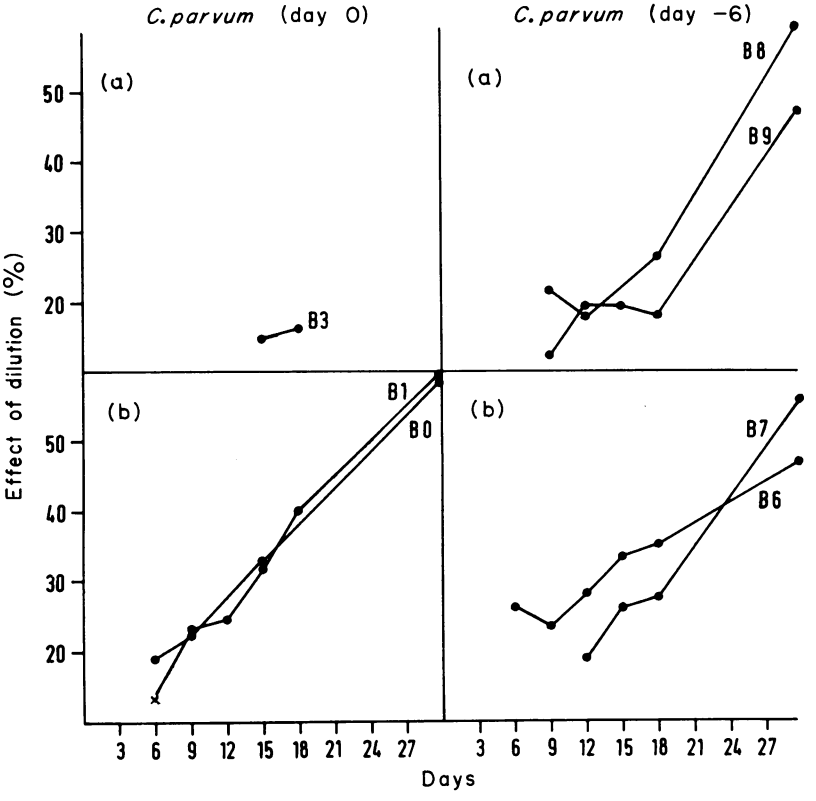

FIG. 2. Primary response: effect of dilution % [(ABC-33, 0·02 μg N BSA/ABC-33, 0·20 μg N BSA) × 100] of *Corynebacterium parvum* (day 0) and *C. parvum* (−6 days) groups given intraperitoneal injections of: (a) CBA or (b) ACBA on day 0. ×, Estimated effect of dilution % from % binding of a 1:10 dilution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

accelerated evolution of the relative binding affinity of the antibodies produced. On days 15 and 18 where ABCs could be determined on rabbit B3, the effect of dilution % of the antisera from this rabbit was lower than rabbits in the other three groups.

Secondary response

On day 30 of the primary response all of the rabbits were given intraperitoneal injections of 10 mg of the bovine serum albumin antigen in the same form as they received for the

primary stimulus and no further *C. parvum*. The decreased amount of antigen compared with that given for the primary stimulus was used in the hope of giving a more sensitive measure of the degree of primary sensitization.

Antigen-binding capacity. The results of the ABC values shown in Fig. 3 were not as had been anticipated. Both groups injected with ACBA did not elicit the expected high anam-

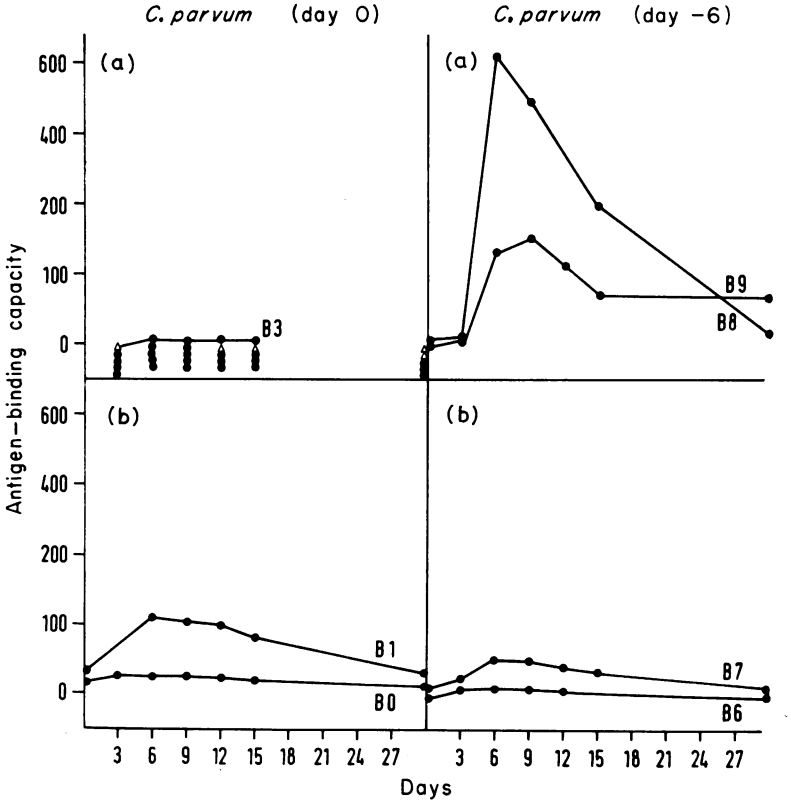

FIG. 3. Secondary response: antigen-binding capacity (Reciprocal ABC-33 end point × 0·20 μg N BSA × 2 × 0·33) of *Corynebacterium parvum* (day 0) and *C. parvum* (−6 days) groups given secondary injections of: (a) CBA or (b) ACBA on day 30 of the primary response (i.e. day 0 of the secondary reponse).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

nestic response, whereas both rabbits which received *C. parvum* on day −6 and CBA on day 0 for the primary stimulus gave a strong secondary response with respect to antigen-binding capacity. The remaining group which received *C. parvum* and CBA on day 0 gave the expected hyporesponsive or unresponsive state when challenged with CBA on day 30.

Effect of dilution. In all of the groups there is in general an immediate, rapid increase in the effect of dilution %. Little comment can be made comparing the effects of dilution as the number of rabbits in each group is small.

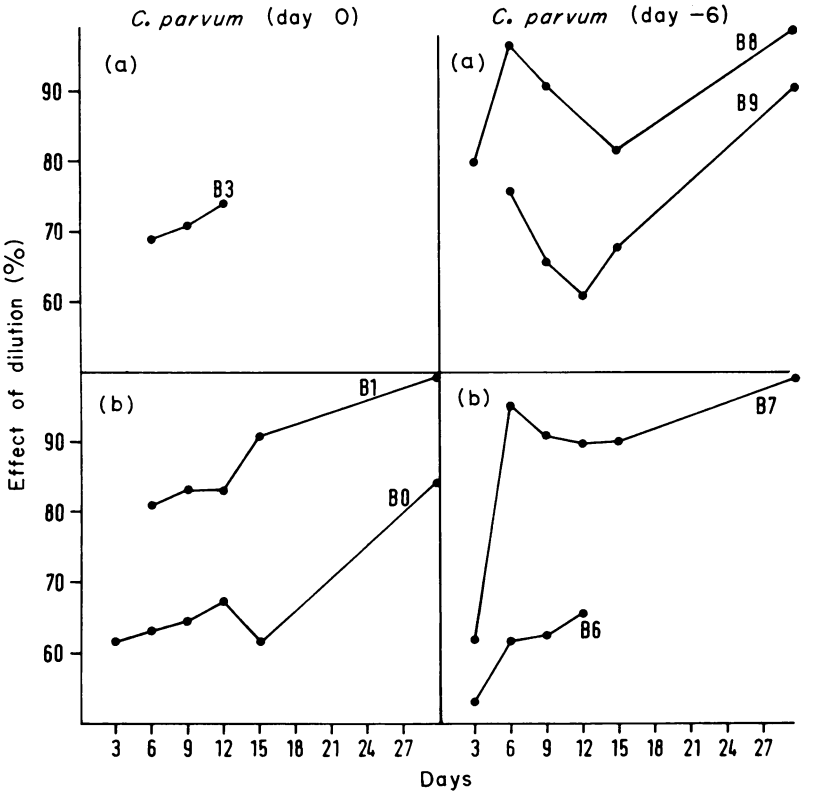

FIG. 4. Secondary response: effect of dilution % [(ABC-33, 0·02 μg N BSA/ABC-33, 0·02 μg N BSA) × 100] of *Corynebacterium parvum* (day 0) and *C. parvum* (−6 days) groups given secondary injections of: (a) CBA or (b) ACBA on day 30 of the primary response (i.e. day 0 of the secondary response).

DISCUSSION

The results of the effects that the physical state of the antigen and lymphoreticular cell proliferation induced by *Corynebacterium parvum* have upon antibody production confirm and extend our previous report and those of Neveu *et al.* (1964), Siskind & Howard (1966) and Biozzi *et al.* (1966). The lack of effect obtained when *C. parvum* was given at the same time as the CBA compared with when the organism was given at −6 days is consistent with the observation of Siskind & Howard (1966); they found augmentation of immunity to SII pneumococcal polysaccharide only when *C. parvum* was injected 7 days beforehand and not when given at the same time as the antigen. It would seem therefore that the adjuvant effects of *C. parvum* can not be entirely explained by the lymphoid hyperplasia or increased phagocytosis which it is known to induce. This view is taken as when CBA and *C. parvum* are injected at the same time one would expect there should be sufficient antigen present 6 days later to stimulate hyperresponsiveness to the antigen which, as we have seen, does not occur. It can be inferred from this that CBA in the absence of 'non-specific factors' seems to cause either unresponsiveness or hyporesponsiveness of the immunologically-competent cells pre-

venting their proliferation as postulated by Claman (1963) and Talmage & Pearlman (1963). Because *C. parvum* is effective when injected before the antigen it either accelerates the 'recognition phase' and/or 'triggering mechanisms' or renders the lymphoid cells less susceptible to tolerance induction. Alternatively, if *C. parvum* is responsible for non-specifically stimulating natural antibody production by causing lymphoid hyperplasia, then the 'anti-BSA antibodies' formed would be valuable for assisting the induction of antibody formation. In contrast to the findings with *C. parvum*, products from other bacteria have been shown to have adjuvant effects when given at the same time as antigen (Munoz, 1964). Claman (1963) demonstrated that the injection of *Salmonella* endotoxin at the same time as unaggregated bovine γ-globulin leads to the production of antibodies compared with unresponsiveness in control animals. It would appear that the effects of *C. parvum* either take place more slowly or that they are altogether different from those of other bacteria.

There is a striking difference between the secondary responses to CBA and ACBA in animals given *C. parvum* at -6 days. A possible explanation for this is that, compared with injection by the intravenous route, intraperitoneally injected ACBA will be removed by the peritoneal macrophages and not come in contact with sensitized lymphoid cells in the spleen and lymph nodes bringing about their stimulation. On the other hand a large proportion of the CBA presumably passes without phagocytosis to the lymphatic system. This is consistent with the observations of Uhr & Baumann (1961) that passively administered antibody given during secondary antigenic stimulation suppresses the immune response by encouraging phagocytosis of the antigen. Furthermore it appears that cultures of primed lymphocytes can be induced to proliferate by direct contact with antigen (see Dutton, 1966). On the other hand for the initiation of the primary response phagocytosis appears to be essential (Fishman, van Rood & Adler, 1965, Mowbray & Scholand, 1966). Perhaps if we had used less ACBA than the 50 mg amounts injected intravenously in the previous report (Pinckard *et al.*, 1967) it might have given similar results on the secondary response to those found here.

The physical state of the antigen and the adjuvant effect of *C. parvum* can be seen from these and the preceding experiments to have marked effects on the magnitude and the increase of the relative binding affinities of the antibodies. This is indirectly supported by the observations of Karush (1962) and Eisen & Siskind (1964) who pointed out that the method of immunization with DNP–bovine γ-globulin affects the magnitude and rate of increase of the average intrinsic association constants of the antibodies produced (see Steiner & Eisen, 1966). Increase in the affinity of antibody during the course of immunization could be explained by the early selective proliferation of those cells capable of producing higher affinity antibody especially if the quantity of antigen available to cause specific lymphoid proliferation is limited.

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