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FACTORS INFLUENCING THE IMMUNE RESPONSE
III. THE BLOCKING EFFECT OF *CORYNEBACTERIUM PARVUM* UPON THE INDUCTION OF ACQUIRED IMMUNOLOGICAL UNRESPONSIVENESS TO BOVINE SERUM ALBUMIN IN THE ADULT RABBIT

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SUMMARY

The intravenous injection of 10 mg aggregate-free, centrifuged bovine albumin into adult rabbits has been shown to induce an unresponsive state to bovine serum albumin (BSA) in the majority of rabbits. An intravenous injection of 15 mg of a heat-killed suspension of *Corynebacterium parvum* either 6 days prior to or simultaneously with centrifuged bovine albumin has been shown to significantly block the induction of unresponsiveness to BSA. Some effects of *C. parvum* upon the lymphoreticular tissues in the rabbit are reported.

INTRODUCTION

Previous reports from our laboratory have shown that the degree of aggregation of bovine albumin influenced not only the quantity of anti-BSA antibody produced in the rabbit but also the rate of increase of the relative binding affinities of the antibodies. Furthermore, we demonstrated that an intravenous injection of a heat-killed suspension of *Corynebacterium parvum* 6 days prior to an intravenous injection of aggregate-free, centrifuged bovine albumin (CBA), which normally led to relatively little or no detectable anti-BSA antibody production, induced a vigorous immune response to BSA characterized by high levels of circulating anti-BSA antibody and a rapid increase of the relative binding affinities of the antibodies produced (Pinckard, Weir & McBride, 1967a, b). In these initial studies only a small proportion of the rabbits failed to produce some detectable antibody to an injection of the aggregate free BSA. In the present study a higher percentage of animals were made unresponsive by using a smaller quantity of antigen and this enabled us to test more clearly whether the injection of CBA induced a specific unresponsive state or the less likely possibility that it was simply sub-immunogenic in aggregate free form. This was achieved by following the response to a challenge dose of alum precipitated BSA. Furthermore, this

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offered the possibility of differentiating between two possible roles of *C. parvum* acting either as a stimulating agent on the antibody producing cells inducing them to make detectable antibody to CBA or actively blocking the induction of a specific unresponsive state by this antigen. Finally in order to assess the general effects of *C. parvum* on the lymphoreticular tissues the effects of *C. parvum* were studied upon: (1) the peripheral white blood cell population, (2) the relative spleen and liver weights, and (3) the histology of the spleen, lymph nodes, liver and lungs.

MATERIALS AND METHODS

Animals

Adult New Zealand White rabbits of both sexes weighing from 2·5 to 3·5 kg were employed.

Antigens

BSA (Cohn Fraction V, Armour Pharmaceutical Lot LNO170) was used throughout the study. CBA and alum precipitated centrifuged bovine albumin (ACBA) were prepared as previously described (Pinckard *et al.*, 1967a).

Preparation of Corynebacterium parvum strain 10387

The procedure for the cultivation, killing and standardization of *C. parvum* is described elsewhere (Pinckard *et al.*, 1967a).

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

The Chloramine-T method of iodination (Hunter & Greenwood, 1962) was employed as previously described (Pinckard *et al.*, 1967a) and the BSA* (crystallized Armour Lot C4508) was standardized by the microKjeldahl method of nitrogen analysis.

Measurement of the antigen-binding capacity

The antigen-binding capacity (ABC) was determined by the ammonium sulphate method of Farr (1958) as described previously (Pinckard *et al.*, 1967a) using 0·02 μg N-BSA* antigen concentration for each serum tested.

Quantitative and differential white blood cell counts

Quantitative white blood cell levels were estimated by the standard procedure in an improved Neubauer counting chamber. Differential counts were performed on blood smears which were stained with Leishman's stain.

Histology

Portions of spleen, mesenteric lymph node, liver and lung were fixed in 10% formalin, embedded in paraffin wax, sectioned and stained with Haematoxylin and Eosin.

Experimental

Three groups of twelve rabbits were employed. The first group received 15 mg (dry weight)

of a heat-killed suspension of *C. parvum* intravenously 6 days prior to an intravenous injection of 10 mg CBA. The second group received 10 mg CBA intravenously followed 30 min later by an intravenous injection of 15 mg (dry weight) of a heat-killed suspension of *C. parvum*. The third control group received only 10 mg CBA, intravenously. All injections were made via the marginal ear vein. The rabbits were bled every 3rd day after the injection of CBA for 30 days. On day 30 of the primary response the rabbits in all three groups were challenged with an intravenous injection of 25 mg ACBA and were bled every 3rd day for another 30 days. All of the rabbit sera were tested for the capacity to bind 0·02 μg N-BSA*. In addition to the above, quantitative and differential white blood cell counts were made on the blood of each rabbit 6 days prior to, the same day, and 6 days after the initial injection (primary) of CBA.

In order to assess the effects which *C. parvum* had upon the lymphoreticular tissue, four rabbits were injected intravenously with 15 mg (dry weight) of the heat-killed suspension of *C. parvum*; 6 days later these rabbits together with four uninjected control rabbits were killed and the weights of their spleens and livers determined. Portions of the spleens, mesenteric lymph nodes, liver and lungs were then obtained for histological examination.

RESULTS

Primary response

The results of the primary responses expressed in the left-hand column of Table 1 show that the majority of the control rabbits injected with 10 mg CBA on day 0 did not produce

TABLE 1

Group	Primary			Secondary		
	R	H	U	R	H	U
C. parvum						
Day −6	7*	0	5	6	1	1
Day 0	9	2	1	10	2	0
Control						
CBA	1	3	8	4	0	8
ACBA	6	0	0			

R, Responsive—positive ABC values; H, hyporesponsive—positive binding; < 33% 0·02 μg N-BSA* bound by a 1:10 serum dilution; U, unresponsive—no detectable binding of 0·02 μg N-BSA* by a 1:10 serum dilution.

* Number of rabbits.

detectable anti-BSA antibody., i.e have the capacity to bind 0·02 μg N-BSA*. Only one rabbit out of twelve produced enough antibody to give a positive antigen-binding capacity (ABC—33); three other rabbits in this group produced low levels of antibody but ABC values could not be determined, i.e. a 1:10 serum dilution bound less than 33% of the 0·20 μg N-BSA* added. The remaining eight rabbits in this group did not produce detectable

anti-BSA antibody. The rabbits which received *C. parvum* simultaneously with CBA responded to a greater degree than the control group just described. Nine out of twelve rabbits produced levels of anti-BSA antibody sufficient to give positive ABC values and two rabbits produced low levels of antibody. Only one rabbit did not produce detectable anti-BSA antibody. The third group, which received *C. parvum* 6 days prior to CBA, appeared to be intermediary between the two other groups. Seven out of twelve rabbits produced sufficient antibody to give positive ABC values and the remaining five rabbits did not produce detectable antibody.

Secondary response

The results of the secondary responses are given in the right-hand column of Table 1. Eight out of the twelve control rabbits, which received only CBA for the primary stimulus, did not produce detectable antibody after a secondary challenge with 25 mg ACBA. The remaining four rabbits in this group produced sufficient antibody to give positive ABC values. To show that ACBA was itself antigenic, six rabbits which had received no prior treatment with CBA or *C. parvum* were injected with 25 mg ACBA intravenously. All six of these animals gave positive ABC values comparable to those pre-treated with *C. parvum* in the primary response to CBA. The secondary response data of the rabbits which received *C. parvum* either prior to or simultaneously with CBA for the primary stimulus differed significantly from the control CBA group just described. Ten out of twelve rabbits which had received *C. parvum* and CBA on day 0 of the primary response gave positive ABC values after secondary challenge with 25 mg ACBA. Two rabbits produced low levels of antibody but ABC values could not be determined. Chi squared analysis using Yates' correction factor for continuity between the control and *C. parvum* (day 0) group and applied to the total number of rabbits which produced detectable anti-BSA antibody after the injection of ACBA as opposed to rabbits without detectable antibody, yielded a value of $\chi^2 = 9{\cdot}18$ ($P<0{\cdot}005$). The results of the remaining group which received *C. parvum* 6 days prior to CBA for the primary stimulus were more difficult to interpret due to deaths which occurred towards the end of the primary response. Sera from six of the surviving eight rabbits gave positive ABC values after secondary challenge with 25 mg ACBA. One rabbit produced low levels of antibody and one rabbit did not produce detectable anti-BSA antibody. Chi squared analysis performed as above between this group and the control group yielded a value of $\chi^2 = 3{\cdot}72$ ($P<0{\cdot}05$). Chi squared analysis between the two *C. parvum* treated groups yielded a value of $\chi^2 = 0{\cdot}044$ ($P<0{\cdot}8$).

Peripheral white blood cell counts

No significant differences in either the total or differential counts were observed in the thirty-six rabbits listed in Table 1 on days -6, 0 and 6 of the primary response. In all three groups there was a small decrease in the numbers of small lymphocytes and a small increase in the numbers of large lymphocytes on day 0 and day 6 of the primary response. The levels of monocytes and polymorphonuclear leucocytes remained constant throughout the test period.

Spleen and liver weights

Spleen and liver weights were determined on four rabbits which had received 15 mg (dry

weight) of a heat-killed suspension of *C. parvum* 6 days prior and compared to four uninjected control rabbits. No differences in liver weights were noted and only a small increase in spleen weight could be detected.

Histology

Spleen, liver and lung sections revealed differences between *C. parvum* treated and control rabbits. The livers of *C. parvum* treated rabbits contained multiple, small, histiocytic granulomata situated in an irregular manner throughout the lobules. A typical granuloma is shown in Fig. 1. Small numbers of mature lymphocytes and plasma cells were present but they were greatly outnumbered by the larger histiocytes.

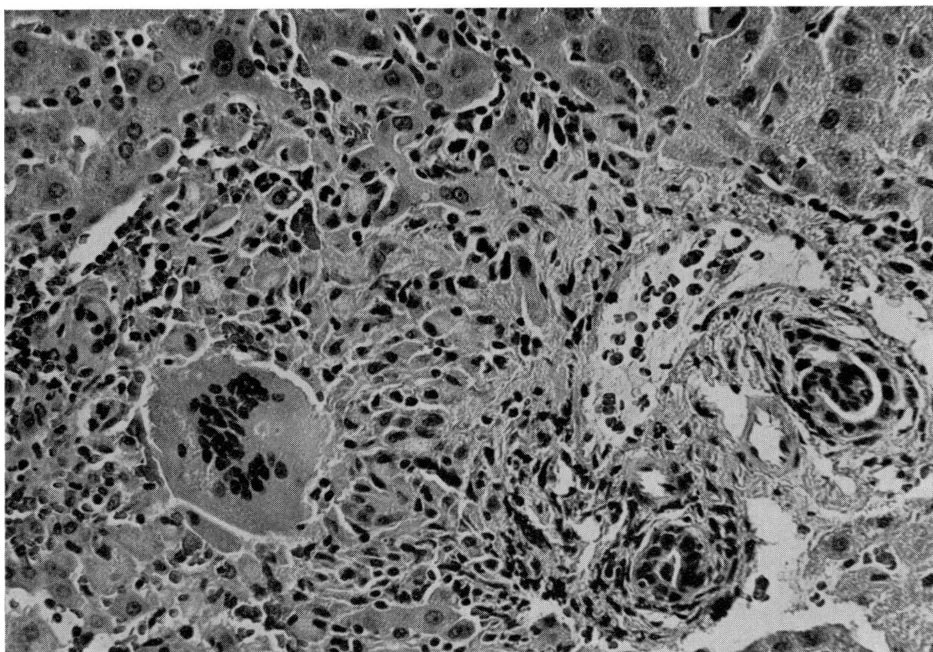

FIG. 1. *Corynebacterium parvum* granuloma in liver. This shows a multinucleate giant cell of the foreign body type. There are numerous histiocytes with round or oval nuclei and abundant eosinophilic cytoplasm. Small numbers of lymphocytes are also present. H & E, ×280.

Lymphocytic foci were also found mainly in and around the portal tracts extending into the sinusoids where they measured up to 100 μ in diameter. No necrosis of liver tissue was observed in any of the sections.

The splenic histology was more difficult to interpret and the only consistent finding was the increase in bulk of the white pulp indicated by the larger size of the Malpighian bodies in the *C. parvum* treated rabbits. The lymph nodes from *C. parvum* treated and control rabbits revealed no differences.

The lungs from *C. parvum* treated rabbits contained granulomata composed of histiocytes

and mononuclear cells with variable numbers of neutrophil polymorphonuclear leucocytes (see Fig. 2). Although never being numerous, the granulomata could be readily distinguished from the foci of lymphoid tissue which are normally present beside the bronchi. These granulomata might have been due to infection in the rabbits, but since they were not found in any of the control rabbits, it would appear that their presence could be ascribed to the lodging of *C. parvum* particles in the lung after intravenous injection.

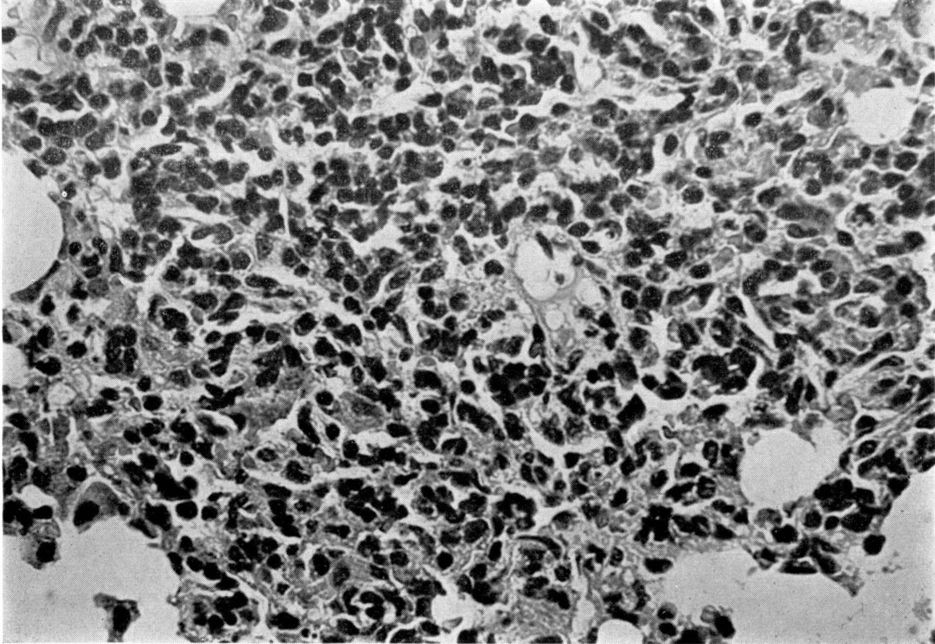

FIG. 2. *Corynebacterium parvum* granuloma in lung. The granuloma consists mainly of histiocytes and lymphocytes with smaller numbers of neutrophil polymorphonuclear leucocytes and plasma cells. H & E, ×360.

DISCUSSION

The present report has demonstrated that acquired immunological unresponsiveness to BSA can be induced in the adult rabbit by a single injection of 10 mg CBA. The unresponsive state could not be terminated 30 days later by an intravenous injection of ACBA. Furthermore, an intravenous injection of a heat-killed suspension of *C. parvum* clearly blocks the induction of the unresponsive state to BSA if administered either 6 days prior to ($P<0{\cdot}05$) or simultaneously with ($P<0{\cdot}005$) 10 mg CBA. No significant difference was seen, however, in the blocking effect between the two *C. parvum* treated groups ($P<0{\cdot}8$). Other workers have reported the blocking of the induction of acquired immunological unresponsiveness in adult animals by various adjuvant materials. Dresser (1962a) found that 'overloading paralysis' to bovine γ-globulin (BGG) in adult mice could be prevented by simultaneous injection of Bacille–Calmette–Guérin (BCG). Claman (1963) reported that the injection

of 100 μg *S. typhosa* endotoxin 2 hr after a tolerogenic dose of aggregate-free BGG blocked the induction of unresponsiveness and led to antibody production. Claman & Bronsky (1965) found that mice pretreated for 2 days with actinomycin-D were less susceptible to the induction of unresponsiveness to aggregate-free BGG. Gery & Waksman (1967) demonstrated that rats, pretreated with zymosan, produced antibody to aggregate-free BGG to a greater degree than control rats. More recently Golub & Weigle (1967a) reported that 25 μg *E. coli* endotoxin inhibited the induction of unresponsiveness to human γ-globulin (HGG) provided the endotoxin was administered the same day or up to 2 days after the injection of aggregate-free HGG.

The adjuvant action of *C. parvum* had been previously thought (Pinckard *et al.*, 1967a, b), like endotoxin, BCG and zymosan, to be related to the extensive lymphoreticular cell proliferation which this organism had been reported to induce. However, the present studies have indicated that *C. parvum* strain 10387 did not cause the profound hepatosplenomegaly reported for strain 936B (Halpern *et al.*, 1964). The French workers reported that strain 936B induced a three-fold increase in spleen weight and a two-fold increase in liver weight. Howard & Inchley (personal communication) found that strain 936B caused a six-fold increase in spleen weight and a three-fold increase in liver weight in mice; however, they found no increase in spleen and liver weight in mice using strain 10387. We have found, however, that *C. parvum* strain 10387 induced histological changes in the livers, lungs and spleens of rabbits. In view of the absent or reduced proliferative effects of *C. parvum* strain 10387 upon the rabbit lymphoreticular tissue in general, it is difficult to postulate possible mechanism(s) of adjuvant action for this micro-organism. Further, the recent work of Golub & Weigle (1967a) with endotoxin indicates that the mechanisms involved in preventing the induction of unresponsiveness to HGG in mice appear to be independent of phagocytic activity of the reticulo-endothelial system. One possibility which has been ruled out is that *C. parvum* might conceivably share antigenic determinants with the BSA molecule. Rabbits which had received 4-monthly intravenous injections of 15 mg *C. parvum* did not produce detectable anti-BSA antibody as judged by the Farr technique using 0·02 μg N-BSA* (Pinckard, Weir & McBride, unpublished results). When these rabbits were later injected with 50 mg BSA they elicited a characteristic primary antibody response indicating that priming towards the BSA molecule had not occurred as a result of prior exposure to *C. parvum*. As no tissue necrosis was observed in any of the histological sections, the possible adjuvant action of *C. parvum* due to the release of tissue breakdown products is unlikely.

It is of interest that *C. parvum* does not act as an adjuvant for antigens which by themselves are good immunogens. *C. parvum* was found to have minimal adjuvant effects for ACBA in rabbits (Pinckard *et al.*, 1967a) and does not augment the immune response to T_4 coliphage in mice (Howard & Inchley, personal communication). In contrast *C. parvum* strain 10387 has powerful adjuvant properties for the relatively weak antigen CBA as demonstrated by the present findings. Further evidence may be provided from the work of Woodruff & Boak (1966) who found that *C. parvum* delayed the appearance of isogenic, mammary tumour formation indicating that *C. parvum* may enhance immunity to weak histocompatibility antigens. In light of recent findings, adjuvant materials in general may enhance antibody formation simply by blocking the induction of unresponsiveness to relatively weak antigenic stimuli.

The mechanism(s) whereby the induction of unresponsiveness to certain antigens in the adult animal are blocked by materials such as *C. parvum* are at present unknown. The

induction of a firm unresponsive state in the adult animal appears to take several days after the injection of the tolerance inducing antigen (Dresser, 1962b; Biro & Garciá, 1965; Gery & Waksman, 1967). Recently Golub & Weigle (1967b) demonstrated by a cell-transfer system that induction of unresponsiveness in adult mice to aggregate-free HGG takes at least 4 days; however, endotoxin was only effective in inhibiting the induction of unresponsiveness if given up to 2 days after the aggregate-free HGG (Golub & Weigle, 1967a). Other materials such as *C. parvum*, actinomycin-D and zymosan can block the induction of unresponsiveness if given prior to the injection of the tolerogenic 'antigen'; endotoxins on the other hand do not block tolerance induction when given prior to the injection of the tolerogenic 'antigen'. The preceding discussion may indicate a complex series of steps leading to an unresponsive state each being sensitive to only certain adjuvant materials; alternatively the various blockers of the unresponsive state may work in the same way but have different rates of adjuvant action. It is of interest to mention that *C. parvum* is unable to block the induction of unresponsiveness to BSA in neonatal rabbits (Pinckard, Weir & McBride, 1967c). Whether this indicates that the induction of unresponsiveness in neonatal and adult rabbits arises through different mechanism(s), or is due to differences in susceptibility of neonatal and adult animals to the induction of unresponsiveness or to the adjuvant action of *C. parvum* can only be a matter of speculation at present. These observations, however, indicate the considerable complexity of the events leading to the induction of acquired immunological unresponsiveness.

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