Clin. exp. Immunol. (1971) **8**, 949–955.

ADJUVANT-INDUCED ANTIRED BLOOD CELL ACTIVITY IN CBA MICE

ANN McCRACKEN, W. H. McBRIDE AND D. M. WEIR

Department of Bacteriology, University of Edinburgh Medical School

(*Received* 14 *September* 1970)

SUMMARY

Various micro-organisms are known to act as immunological adjuvants and included amongst these are Corynebacteriaceae. Numerous studies on *Corynebacterium parvum* have shown, in particular, its ability to cause proliferation and enhanced activity of the reticulo-endothelial system. This organism also leads in mice to anaemia and this report describes the simultaneous appearance of a red cell autoantibody in mice injected with *C. parvum* or another diphtheroid (SF 16) isolated from rheumatoid joint fluid. The significance of this latter observation is considered in relation to the unexplained anaemia of rheumatoid arthritis.

INTRODUCTION

Various Corynebacteriaceae have been shown to possess adjuvant activity under a variety of circumstances (Halpern *et al.*, 1966; Smith & Woodruff, 1968; Paronetto, 1970; Nussenzweig, 1967). They are also capable in varying degrees of inducing lymphohistiocytic proliferation and enhanced phagocytosis by the reticulo-endothelial system (Prévot & Van Phi, 1964). Amongst these organisms *C. parvum* has received considerable attention and we have reported a preventive effect upon the experimental induction of immunological tolerance (Pinckard, Weir & McBride, 1968). During our studies with this organism and another diphtheroid (SF 16) with similar adjuvant activity we noted that injection of a heat-killed suspension of either of these organisms into CBA mice resulted in an increase in the number of spleen cells with activity directed against normal mouse red blood cells. The tests employed were the Jerne plaque technique, in which antibody produced by spleen cells brings about the *in vitro* lysis of red cells in a semi-solid medium, and immuno-cyto-adherence, where spleen cells producing antibody form rosettes of attached red cells. The organism SF 16 was isolated from synovial fluid of a patient with rheumatoid arthritis (Stewart, Alexander & Duthie, 1969). Since an unexplained anaemia is present in rheumatoid

Correspondence: Dr D. M. Weir, Department of Bacteriology, University of Edinburgh Medical School, Edinburgh.

arthritis (Alexander & Duthie, 1962), and since *C. parvum* has been shown to lead to a decrease in haemoglobin levels and total red blood cells (Halpern & Fray, 1969), it was decided to further quantitate the antimouse red cell response produced in mice by these organisms, particularly SF 16, in the hope of throwing some light on the mechanisms of the anaemia associated with rheumatoid arthritis.

MATERIALS AND METHODS

Microorganisms. The SF 16 organism was kindly provided by Dr Sheila Stewart and was cultured in PPLO broth medium containing 'Tween 80' (Atlas Chemical Industries Inc.). *C. parvum* strain 10387 was obtained from the N.C.T.C., Colindale, grown in cooked gigot broth. Both organisms were washed ten times in sterile saline, heat-killed, divided into aliquots and stored at −20°C.

Animals. Young adult CBA mice were used.

Spleen cell suspension. Whole spleens were gently homogenized in Dutton's saline (Golub *et al.*, 1968) and sieved through fine mesh, washed once and resuspended to 1×10^7 cells/ml.

Red blood cell suspension. Red blood cells from stock CBA mice were collected in Alsever's solution and washed in Dutton's saline four times.

Agar plaque technique. The technique used was a modified version of Dresser & Wortis (1967). 0·1 ml of 20% washed mouse red blood cells and 0·1 ml of spleen cell suspension were added to 1·8 ml of 0·7% (w/v) Noble agar containing DEAE dextran (50 mg/100 ml agar) at 48°C. This mixture was poured into a Petri dish containing a bottom layer of agar and incubated for 2 hr after which a 1/10 dilution of guinea-pig serum was added for 45 min (37°C). The plaque-forming cells (PFC) were counted under × 30 magnification. Control plates without spleen cells and plates receiving inactivated complement were incorporated into each experiment.

Immuno-cytoadherence test. This was based on a modification of the method of Biozzi *et al.* (1966). Equal volumes (0·5 ml) of red blood cells and spleen cells in the proportion of 10:1 were mixed together and centrifuged at 150 *g* for 7 min. They were incubated for 2 hr at 4°C and gently resuspended using a Pasteur pipette. The number of rosette-forming cells in a 1/10 dilution of trypan blue was counted in an improved Neubauer haemocytometer.

RESULTS

The number of PFCs and RFCs were first estimated after intraperitoneal injection of 2·3 mg (dry weight) *C. parvum* organism in saline. Tables 1 and 2 show that both tests can detect a resulting increase in the proportion of spleen cells reacting with their isologous red cells.

Similar, more detailed, experiments have been performed using the organism SF 16 isolated from rheumatoid joint material. Fig. 1 shows that doses of 0·04–3 mg (dry weight) organisms were all capable of stimulating the appearance of PFCs. The duration of the response was estimated using a single dose of 2·3 mg SF 16, Figs. 2 and 3; after reaching a peak at 5–7 days the response gradually falls off over the next 7–10 days. The RFCs tend to rise slightly earlier than the PFCs and fall off more rapidly. This response was not due to contaminating culture medium as animals injected with the washings from uninoculated plates gave the same background results as saline injected control animals.

TABLE 1. Plaque forming cells (PFCs) with CBA mouse spleen cells and isologous red cells after injection of 2·3 mg *C. parvum*; each mean was obtained from a group of ten animals

Days after injection		Log_{10} mean plaques/10^6 cells	Standard error 95% confidence limits
2	*C. parvum*	1·0745	±0·1591
	Control	0·6171	±0·2582
4	*C. parvum*	1·6107	±0·2378
	Control	0·7405	±0·2355
6	*C. parvum*	1·0801	±0·1877
	Control	0·6612	±0·2406
7	*C. parvum*	1·0140	±0·3121
	Control	0·5001	±0·2146
10	*C. parvum*	1·5576	±0·1059
	Control	0·4912	±0·2224
14	*C. parvum*	0·5827	±0·4600
	Control	0·6014	±0·2814
18	*C. parvum*	0·8381	±0·2627
	Control	0·6142	±0·2613

TABLE 2. Rosette forming cells (RFCs) with CBA mouse spleen cells and isologous red cells after injection of 2·3 mg *C. parvum*; each mean was obtained from a group of ten animals

Days after injection		Mean rosettes/10^3 spleen cells	Standard error 95% confidence limits
2	*C. parvum*	11·83	±4·29
	Control	5·75	±3·27
4	*C. parvum*	14·80	±3·67
	Control	6·14	±2·92
6	*C. parvum*	24·60	±1·94
	Control	5·25	±2·14
7	*C. parvum*	15·20	±3·24
	Control	5·40	±3·14
10	*C. parvum*	8·00	±3·20
	Control	7·25	±3·24
14	*C. parvum*	7·14	±2·89
	Control	5·24	±1·94
18	*C. parvum*	8·00	±2·20
	Control	3·70	±2·97

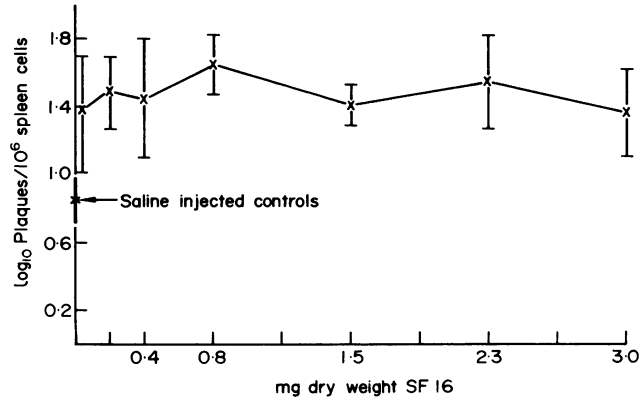

FIG. 1. Plaques 5 days after various doses of SF 16 organism. Each point represents the mean obtained with six mouse spleens and shows the 95% confidence limits. Each test was performed in triplicate.

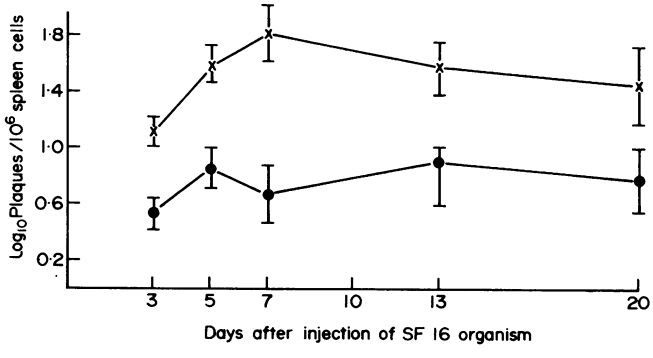

FIG. 2. Plaques at intervals after 2·3 mg of SF 16 organism. Each point represents the mean obtained with six mouse spleens and shows the 95% confidence limits. Each test was performed in triplicate. ×, SF16 injected; ●, saline injected.

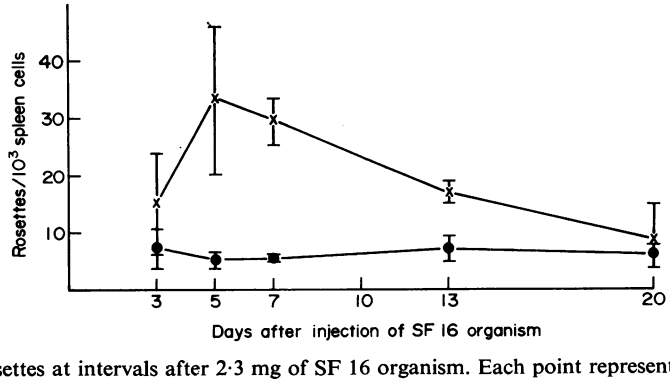

FIG. 3. Rosettes at intervals after 2·3 mg of SF 16 organism. Each point represents the mean obtained with six mouse spleens and shows the 95% confidence limits. Each test was performed in triplicate. ×, SF16 injected; ●, saline injected.

The effect of injecting *C. parvum* and SF 16 on the haemoglobin levels of CBA mice is shown in Table 3. Both organisms bring about a fall in haemoglobin level compared with saline injected control animals which has not returned to normal levels by 14 days.

TABLE 3. Effect of injection of *C. parvum* (2·3 mg to eight mice) and SF 16 (2·3 mg to twenty-one mice) on haemoglobin levels in CBA mice; results expressed as per cent of saline injected control animals

Days after injection	C. parvum treated	SF 16 treated
1	100	100
2	100	100
3	100	71·3
5	94·7	79·8
7	93	79·3
10	88	82·9
14	77·4	80·8

DISCUSSION

Injection of CBA mice with both *C. parvum* and the SF 16 organism can be seen to result in a short-lived increase in the numbers of spleen cells making antibody to CBA red cells. At the same time there is a corresponding fall in haemoglobin levels which persists for more than 2 weeks.

Injection of *C. parvum* is known to be associated with a fall in haemoglobin and total red blood cell levels in C57Bl and NZB mice. In addition, some animals of both strains also developed antired cell antibodies as detected by the Coombs test although the response is more marked in the NZB animals (Halpern & Fray, 1969). In the present studies a fall in haemoglobin levels was first detected between 3 and 5 days after injection, coincident with the rise in RFCs. In the absence of any evidence for direct red cell haemolysis by these organisms *in vivo* and *in vitro* other explanations must be sought for the observed effects.

At present we are not able completely to rule out the possibility that *C. parvum* and SF 16 share common antigenic determinants with mouse red blood cells. This, however, seems unlikely as neither mouse nor rabbit antisera to these organisms show agglutinin activity against red blood cells (McCracken, unpublished results).

Whether or not the presence of antired blood cell activity is the cause of or even an influence on the haemolytic process is uncertain. In view of the stimulating effect of *C. parvum* on phagocytosis (Prévot & Van Phi, 1964), it seems possible that there is a more rapid ingestion of red cells by 'stimulated' macrophages and this might account for the anaemia. In support of this possibility the RES stimulant Zymosan has been shown to be capable of inducing anaemia in mice (Gorstein & Benacerraf, 1960). No evidence of antired cell antibody was found by the Coombs test.

Another explanation which we favour for the observed results may lie in the nonspecific stimulation of a small number of pre-existing antibody-forming clones. Bacterial endotoxin injection alone has been shown to increase the number of background haemolysin-forming

cells against heterologous red blood cells, as also have substances released during the interaction of antigens with cells from animals immunized with the antigen (Braun & Nakano, 1964). It seems possible that substances released by lymphoid cells in the course of the normal powerful humoral or cell-mediated response to *C. parvum* and SF 16 may lead to non-specific proliferation of pre-existing antibody-forming cells. We have not yet determined in detail whether other lymphoreticular stimulatory agents can produce the same effects as *C. parvum* although preliminary data suggest that mycoplasma pneumoniae (6 mg of BHF strain) and BCG (6 mg) show at best a much smaller, less consistent effect. *B. pertussis* in a dose of 1×10^{10} organisms has a rather less marked effect on the number of antimouse red cell plaques than SF 16.

At present it is a matter of speculation whether this type of autoimmune response has any pathogenic significance. The anaemia of rheumatoid arthritis has the characteristics of the anaemia associated with chronic infection (Alexander & Duthie, 1962). The antired cell response after the treatment described in this work warrants further consideration of a mechanism of this type, possibly induced by micro-organisms, as a direct or indirect cause of anaemia in rheumatoid arthritis, particularly with reference to the diphtheroids of similar characteristics to SF 16 isolated from patients with this disease, by Stewart *et al.* (1969) and Hill *et al.* (1967).

ACKNOWLEDGMENTS

This work was supported by grants from the Medical Research Council and the Arthritis and Rheumatism Council. Dr Sheila Stewart provided the SF 16 organism and Professor B. P. Marmion the mycoplasma pneumonia strain.

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