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The role of macrophages in the adjuvant effect on antibody production of *Corynebacterium parvum*

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SUMMARY

Spleen cells from mice pre-treated with *C. parvum* gave an enhanced *in vitro* antibody response to SRBC, but not to DNP–POL. This enhancing activity was associated with the adherent, but not the non-adherent spleen cell population and was found to be radioresistant. It is concluded that macrophages are directly involved in the adjuvant effect of *C. parvum* and the possible mechanisms of action are discussed.

INTRODUCTION

Administration of *C. parvum* to experimental animals has a marked adjuvant effect on antibody responses to various antigens, although cell-mediated immunological reactions have been found on several occasions to be depressed (reviewed by Scott, 1974). *C. parvum* is also a potent activator of the mononuclear phagocyte system, stimulating both cellular proliferation (Warr & Šljivić, 1974a) and phagocytic activity *in vivo* (Halpern *et al.*, 1964; Warr & Šljivić, 1974b). It has been proposed that the adjuvant effect of *C. parvum* on antibody production is mediated via these stimulated macrophages (Howard, Scott & Christie, 1973) and the experiments we describe here demonstrate this.

MATERIALS AND METHODS

Mice. CBA female mice aged between 3 and 9 months were used as donors of spleen cells. Experimental mice were injected intravenously with 0·2 ml of *C. parvum* suspension containing 1·75 mg dry weight of organisms per millilitre (Batch PX 289, Wellcome Research Laboratories, Beckenham, Kent, England) 5 days before their spleens were removed. Control mice of similar age were untreated.

Preparation and culture of spleen cells. The medium used throughout was RPMI 1640 with glutamine (Flow Laboratories Limited, Irvine, Scotland), containing 5% heat-inactivated foetal calf serum, 15 mM HEPES, 24 mM sodium bicarbonate, 125 mg/l ampicillin, 125 mg/l cloxacillin and 10,000 i.u./l gentamicin. Spleen cell suspensions were prepared by sieving through a fine metal sieve. The cells were washed once and resuspended at a concentration of 20×10^6/ml. The cells were fractionated into non-adherent and adherent by incubating 15–20 ml of the suspension in a 300-ml glass medical flask lying flat on its side. After 30 min at 37°C the flask was turned over and the cells incubated for another 30 min. The non-adherent cells were then gently agitated and poured off, after which the surface was washed vigorously with fresh medium and the adherent cells removed from glass by means of a rubber policeman. The non-adherent cell fraction obtained in this way contained approximately 70% of the original cell concentration.

The cells were cultured for 4 days in Marbrook chambers (Marbrook, 1967) in the presence of either 2×10^6 sheep erythrocytes (SRBC) or 0·1 μg DNP–POL (kindly provided by Dr M. Feldmann), in a volume of 1 ml. Unfractionated spleen cells were cultured at a concentration of 20×10^6/ml and non-adherent ones at

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a concentration of 13–14 × 10^6/ml. Adherent cells were added to some cultures at the rate of 2–3 × 10^6 viable cells.

For some experiments cells were irradiated *in vitro* with a dose of 600 R using a Siemens Stabilipan X-ray machine operating at 240 kV and 10 mA, with a target distance of 34 cm, 1 mm Cu filter and a dose rate of 91·1 R/min.

The antibody response in the cultures was measured in terms of plaque-forming cells (PFC) by the method of Cunningham (Cunningham & Szenberg, 1968), using either untreated SRBC or DNP-coated SRBC for the response to DNP–POL (Strausbach, Sulica & Givol, 1970). The results are given as means ± s.e. PFC/culture of quadruplicate cultures of spleen cells pooled from five to seven mice.

RESULTS

The results of three separate experiments using SRBC as antigen and one experiment using DNP–POL are summarized in Table 1, which has been subdivided into sections for ease of reference. The results of some control cultures are not included in the Table. These showed that the numbers of anti-SRBC PFC of unfractionated spleen cells cultured in the absence of SRBC were 104 ± 30 and 164 ± 47 for normal and *C. parvum*-treated mice, respectively. No PFC were found in cultures containing only adherent cells and either of the two antigens.

Experiments using normal spleen cells (section A) confirmed earlier findings that macrophages were required for the *in vitro* response to SRBC (Mosier, 1967; Sjöberg, Andersson & Möller, 1972), but not to DNP–POL (Diener, Shortman & Russell, 1970).

Cultures of unfractionated spleen cells from *C. parvum*-treated mice produced approximately three times the number of anti-SRBC PFC found in cultures of cells from untreated

TABLE 1. Effect of pre-treatment of mice with *C. parvum* on the antibody response of spleen cells *in vitro*

Section	Spleen cells in culture			Response to:			
				SRBC			DNP–POL
	Unfractionated	Non-adherent	Adherent	1	2	3	
A	Normal	—	—	2050 ± 71	2190 ± 110	2280 ± 50	1446 ± 64
	—	Normal	—	210 ± 13	140 ± 22	225 ± 22	1236 ± 81
	—	Normal	Normal	1861 ± 62	1676 ± 54	1776 ± 17	1200 ± 90
B	*C. parvum*	—	—	7200 ± 423	6780 ± 100		1450 ± 68
	—	*C. parvum*	—	290 ± 55	110 ± 24		1486 ± 62
	—	*C. parvum*	*C. parvum*		5856 ± 210		1566 ± 76
C	—	Normal	*C. parvum*	3310 ± 273	5536 ± 70		1616 ± 87
	—	*C. parvum*	Normal	1816 ± 59	1820 ± 17		1536 ± 43
D	Normal-X	—	—			125 ± 33	
	—	Normal-X	—			0	
	—	Normal-X	Normal-X			0	
	—	Normal-X	Normal			20 ± 8	0
	—	Normal	Normal-X			1700 ± 134	1230 ± 53
E	—	*C. parvum*-X	*C. parvum*		140 ± 20		0
	—	*C. parvum*	*C. parvum*-X		5520 ± 70		1656 ± 76
F	—	*C. parvum*	Normal-X		1726 ± 35		1566 ± 76
	—	Normal-X	*C. parvum*		40 ± 18		0
	—	*C. parvum*-X	*Normal*		106 ± 30		0
	—	Normal	*C. parvum*-X		5610 ± 138		1600 ± 110

Normal indicates spleen cells from untreated mice and *C. parvum* indicates spleen cells taken from pre-treated mice. The suffix X indicates that cells were exposed to 600 R X-irradiation *in vitro*. The results are given as means ± s.e. PFC per culture of quadruplicate cultures.

mice (section B *vs* A), thus demonstrating the adjuvant effect of *C. parvum*. Removal of adherent cells from *C. parvum* spleen cell suspensions reduced the PFC response to background level and reconstitution with these cells restored almost completely the enhanced response. The involvement of macrophages in the enhanced responce to SRBC was demonstrated in recombination experiments in which non-adherent and adherent spleen cell fractions from untreated and pre-treated mice were cultured (section C). The PFC response was enhanced in cultures containing normal lymphocytes and *C. parvum* macrophages, but not in those containing *C. parvum* lymphocytes and normal macrophages.

Experiments in which irradiated spleen cells or cell fractions were cultured in the presence of SRBC provided further evidence that the adjuvant property was associated with the adherent cell fraction from *C. parvum*-treated mice (sections E and F). A dose of 600 R, which abolished the response of unfractionated spleen cells and the response of the non-adherent cell fraction in the presence of unirradiated adherent cells, had no effect on the co-operative function of normal adherent cells or the enhancing activity of these cells after administration of *C. parvum*.

The PFC response to DNP–POL was not affected by the removal of adherent cells or by the pre-treatment of donor mice with *C. parvum*. This response was, however, completely abolished when the non-adherent spleen cells were irradiated.

DISCUSSION

The experiments described here are relevant to several aspects of the adjuvant action of *C. parvum* on antibody production:

(1) Spleen cells from animals pre-treated with *C. parvum* when cultured *in vitro* in the presence of SRBC, a thymus-dependent antigen (Claman & Chaperon, 1969), gave an enhanced antibody response. In this respect *C. parvum* is different from *Bordetella pertussis* and BCG vaccines which produced an enhancement of the response to SRBC only when these adjuvants were present in the cultures of spleen cells from adjuvant-primed mice (Maillard & Bloom, 1972).

(2) The adjuvant effect was associated with the adherent spleen cell population, presumably macrophages, and not with the non-adherent cells, i.e. lymphocytes. The finding that the adherent cells were radioresistant, in contrast to the non-adherent cells, supports the conclusion that they were macrophages (Sjöberg *et al.*, 1972). The experiments in which irradiated cells were used also suggest that administration of *C. parvum* did not confer the property of glass adherence upon the radiosensitive cells (i.e. lymphocytes) and that the ability of macrophages to enhance the antibody response was not associated with their proliferation, since this is radiosensitive (Warr & Šljivić, 1974a).

(3) The response to DNP–POL, a T cell-independent (Feldmann & Basten, 1971), and macrophage-independent (Diener *et al.*, 1970) antigen, was not influenced by the pre-treatment of spleen cell donors with *C. parvum* under the conditions of the present experiments. This would suggest that administration of *C. parvum* did not influence, directly or indirectly, the function of B lymphocytes, which is at variance with *in vivo* findings using other T cell-independent antigens (Howard, Christie & Scott, 1973).

Although these experiments demonstrate that macrophages play a crucial role in the manifestation of the adjuvant effect after administration of *C. parvum*, the mechanisms involved are not understood. On the basis of the known involvement of macrophages in other systems the following possibilities could be considered at present.

(a) Macrophages from *C. parvum*-treated animals may be exerting a potentiating effect on antibody production because they are directly stimulated by ingested *C. parvum* organisms and take up, degrade and present the immunogenic moiety of the antigen in a more efficient manner. A difference in antigen handling has been found, for example, in Biozzi

'high' and 'low' responder mice and this is thought to relate to the different ability of these animals to produce antibodies (Wiener & Bandieri, 1974).

(b) The effect of *C. parvum* on macrophages may be mediated by T lymphocytes as has been suggested in the case of other adjuvants (Allison & Davies, 1971; Maillard & Bloom, 1972), perhaps through a non-specific factor released by T cells (Waldmann, 1975), although it has been suggested that such a factor does not require macrophages for its function (Lefkovits *et al.*, 1975). The present results are not incompatible with this possibility, providing it is postulated that (i) the continuous presence of activated T cells is not required, since macrophages from *C. parvum*-treated animals enhanced the response of normal lymphocytes, and (ii) activated T cells cannot stimulate macrophages under culture conditions, since lymphocytes from pre-treated animals did not show enhancement in the presense of normal macrophages. However, *in vivo* studies have revealed that *C. parvum* had an adjuvant effect on the response to SRBC in T cell-deprived mice (Howard *et al.*, 1973) and the response to pneumococcal polysaccharide, a thymus-independent antigen (Howard *et al.*, 1973), but not on the response to SRBC in thymus-less (nude) mice (Warr & James, 1975). In addition, *C. parvum* stimulated the phagocytic activity in T cell-deprived mice (Woodruff, McBride & Dunbar, 1974) and generated macrophages cytotoxic to tumour cells both in these and nude mice (Ghaffar, Cullen & Woodruff, 1975).

(c) Macrophages stimulated by *C. parvum* may be exerting their effect on immunologically competent cells through some soluble factor(s). Although no evidence is available at present to support such a mode of action, it has been shown recently that macrophage products can stimulate or depress the proliferation of lymphoid cells *in vitro* (Calderon & Unanue, 1975). If a similar mechanism is to be postulated to explain the effect of *C. parvum* it will have to act on T lymphocytes, since the response to DNP–POL was unaffected.

Further experiments are now in progress in an attempt to elucidate which of the above possible mechanisms may be operative.

NOTE ADDED IN PROOF

Similar results, using peritoneal macrophages from *C. parvum*-treated mice, have recently been described (Wiener, E. (1975) *Cell. Immunol.* **19,** 1), and a difference in the handling of KLH by these cells has been found (Wiener, E. & Bandieri, A. (1975) *Immunology*, **29,** 265).

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