Immunomodulation by Myxospores of *Myxococcus xanthus*

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Glycerol-induced myxospores of *Myxococcus xanthus* caused non-specific modulation of humoral and cellular immune responses in laboratory animals. The number of cells which formed specific haemolysins in spleens of mice immunized with sheep erythrocytes was increased when 0.5×10^8 myxospores were inoculated 2 d after the erythrocytes, and decreased when myxospores were injected 2 d before or at the same time as the erythrocytes. Both the IgG primary response and the secondary response to erythrocytes were decreased in rabbits after pretreatment with 2×10^8 myxospores per rabbit. Delayed-type hypersensitivity to sheep erythrocytes was also suppressed in mice after intraperitoneal (i.p.) injection of 0.3×10^8 myxospores. One day after i.p. injection of myxospores, neither an inflammatory response nor bone marrow cell depletion was observed in mice. These results support the idea that *M. xanthus* myxospores possess diverse immunomodulation properties apparently due to factors different from the classical LPS of Gram-negative bacteria.

INTRODUCTION

Cells of a number of microbial species as well as various bacterial products possess immunomodulating properties (reviewed by White, 1976; Stewart-Tull, 1980).

The fruiting myxobacteria comprise a group of closely related Gram-negative bacteria which are typified by a life cycle quite remarkable in view of their prokaryotic nature. Under certain conditions the cells aggregate and cooperatively construct macroscopic fruiting bodies within which the individual cells convert to myxospores (Dworkin, 1966). Myxospore formation can be induced in liquid culture by a variety of techniques (Dworkin & Gibson, 1964; Burchard, 1975).

In a previous work, we demonstrated that an induced myxospore suspension of *Myxococcus xanthus* modulated the humoral response to sheep red blood cells (SRBC) in mice, whereas vegetative cells failed to do so (Ruiz *et al.*, 1984). It appears that the existence of different immunomodulation factors in the vegetative cells and in the myxospores of *M. xanthus* resembles the situation in *Bacillus megaterium* (Ruiz-Bravo *et al.*, 1981).

The aim of this work was to study the possible immunomodulation effect of induced myxospores of *M. xanthus* CCM 3494 on several parameters of immunity.

METHODS

Animals. Male Balb/c mice were bred in our laboratory from breeding pairs originally obtained from the Department of Immunology, London Hospital Medical School, UK. Experimental groups were made by random selection of 20–25 g animals 6–8 weeks of age.

Rabbits weighing 2.5–3.0 kg were purchased locally.

Preparation of bacteria. *Myxococcus xanthus* CCM 3494 was grown with aeration at 30 °C in liquid medium containing 1% (w/v) Casitone (Difco) and 0.2% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Myxospore formation was induced by the

Abbreviations: SRBC, sheep red blood cells; PFC, splenic haemolytic plaque-forming cells; DTH, delayed-type hypersensitivity; 2ME, 2-mercaptoethanol; i.p., intraperitoneal(ly); i.v. intravenous(ly); s.c. subcutaneous(ly).

addition of glycerol to a final concentration of 4.6% (w/v) as described by Dworkin & Gibson (1964). Myxospores were washed, suspended in phosphate-buffered saline (PBS) (0.85% NaCl in 0.15 M-sodium phosphate, pH 7.2), and concentrated to a density of 2.5×10^8 myxospores ml^{-1} . The total cell number was determined using a Petroff-Hausser counting chamber.

Antigen. SRBC were washed three times, resuspended in PBS and stored at 4 °C until needed.

Antibody-forming cell assay. Mice were injected i.p. with 0.2 ml of the myxospore suspension (0.5×10^8 myxospores) (i) 2 d before, (ii) the same day as, or (iii) 2 d after i.p. immunization with 1×10^9 SRBC. Mice were killed 4 d after SRBC injection and splenic haemolytic plaque-forming cells (PFC) were counted by the technique of Cunningham & Szenberg (1968).

Serum antibody assay. Rabbits were injected i.p. or intravenously (i.v.) with 0.8 ml of the myxospore suspension containing 2.5×10^8 myxospores ml^{-1} . Control animals received 0.8 ml sterile PBS. All animals were injected i.p. with 2 ml 10% (v/v) SRBC in PBS, 4 and 26 d after inoculation with the myxospore preparation.

Blood samples were withdrawn from the marginal ear vein at various time intervals; after coagulation and clot separation, serum titres were determined. For the haemagglutination assay, twofold dilutions of serum in PBS were prepared in microtitre plates with round-bottomed wells. A 50 μl sample of a 0.5% (v/v) SRBC suspension was added to 50 μl of the diluted serum. To determine the titre of antibodies resistant to 2-mercaptoethanol (2ME), serum was preincubated with 1.4% (v/v) 2ME at 37 °C for 1 h (Yashphe, 1972). Haemagglutinating titres were determined after 4 h in all tests.

Assay with peritoneal cells. Mice were killed 24 and 72 h after i.p. inoculation with 0.2 ml of the myxospore suspension. Both peritoneal cell harvest and culture were performed as described previously (Fishel *et al.*, 1976; Yarkoni *et al.*, 1977), with minor modifications. Briefly, mice received i.p. 3 ml of medium 199 with 10% (v/v) heat-inactivated calf serum and sodium heparinate. Peritoneal fluid was withdrawn, centrifuged, and the sediment resuspended in fresh medium. Viable cells were counted and suspensions distributed into plastic Petri dishes. After 2 h incubation at 37 °C, cells which failed to adhere were removed and counted. For the phagocytosis assay, the adherent cell monolayers were washed with fresh medium containing 10^8 SRBC. After incubation at 37 °C for 1 h, the monolayers were washed, fixed and stained with Giemsa stain. The number of phagocytic cells were counted among at least 200 adherent cells.

Collection and quantification of bone marrow cells. Bone marrow cells were collected by dissecting one or both femurs of each mouse, cutting the bone at the metaphysis, and flushing the marrow cavity with 1.0 ml medium 199 (with heparin) using a 27-gauge needle. The cell suspension was centrifuged at 400 *g* for 10 min and the sediment was resuspended in 2 ml medium 199. Viable cells were counted by the Trypan blue exclusion method.

Delayed-type hypersensitivity (DTH) determined by footpad swelling. As a pretreatment, mice were injected subcutaneously (s.c.) or i.p. with a mixture of 0.125 ml SRBC (2%, v/v, in PBS) and 0.125 ml of the myxospore suspension. Control mice received only 0.25 ml of a 1% (v/v) suspension of SRBC in PBS, in the same way as the treated mice. After 7 d, mice were injected in the left hind footpad with 0.05 ml SRBC (1%, v/v, in PBS), and in the right hind footpad with 0.05 ml PBS as the control. The thickness of the stimulated and the control footpads was measured with a dial-gauge caliper 24 h after the elicitation. The difference in footpad thickness was expressed in units of 0.1 mm.

Statistics. The differences between mean results in animals treated with myxospores of *M. xanthus* and controls were analysed by Student's *t*-test. Statistical significance was determined at the $P < 0.05$ level.

RESULTS

Modulation of the PFC response by M. xanthus myxospores. When myxospores were administered to mice 2 d before or on the same day of immunization with SRBC, the PFC response was suppressed (Fig. 1). In contrast, the injection of myxospores 2 d after SRBC significantly enhanced the PFC response.

Effect of M. xanthus myxospores on haemagglutination titres to SRBC. Haemagglutination titres of the serum of rabbits pretreated with *M. xanthus* myxospores are shown in Fig. 2. The primary response was enhanced by myxospores injected i.v. However, pretreatment with myxospores resulted in suppression of the secondary response, irrespective of the route of inoculation. Qualitative changes in the primary response were caused by both i.v. and i.p. injection of myxospores. Thus, the amount of 2ME-labile antibodies (IgM) increased while the amount of 2ME-resistant antibodies (IgG) decreased as a result of pretreatment of the animals.

Peritoneal leucocyte response to the inoculation of M. xanthus myxospores. The effect of pretreatment with myxospores on the peritoneal cell populations is shown in Table 1. A

Fig. 1

Fig. 1. Modulation of the PFC response to SRBC in mice by myxospores of *M. xanthus*. □, Control mice; ■, myxospore-treated mice. The values are means of results from at least six mice $\pm$ 95% confidence limits.

Fig. 2

Fig. 2. Effect of pretreatment with myxospores of *M. xanthus* on the primary and secondary serum antibody response to SRBC in rabbits. (a) Control rabbits (saline on day 0); (b) treated rabbits (2×10^8 myxospores per rabbit on day 0 injected i.v.); (c) treated rabbits (2×10^8 myxospores per rabbit on day 0 injected i.p.). ●, Total haemagglutinating titre; ■, 2ME-resistant haemagglutinating titre. The results are means of five experiments $\pm$ 95% confidence limits.

Table 1. Peritoneal cell response to *M. xanthus* myxospores in mice

Pretreatment	Killed on day	$10^{-6} \times$ No. of peritoneal cells*	Adherent cells (%)	Adherent phagocytes	
				Percentage†	Phagocytic index‡
Saline	1	6.4 ± 2.4	74.1 ± 7.0	18.4 ± 1.5	1.2 ± 0.1
Myxospores§	1	6.7 ± 1.3	69.8 ± 8.1	24.0 ± 5.2	$2.7 \pm 0.1 $
Saline	3	8.2 ± 1.2	80.1 ± 7.3	18.5 ± 1.5	1.2 ± 0.1
Myxospores§	3	$10.3 \pm 1.0 $	78.4 ± 1.5	$28.3 \pm 4.7 $	$2.9 \pm 0.4 $

* Arithmetic means of results from at least six mice $\pm$ 95% confidence limits.

† Percentage relative to the total number of adherent cells.

‡ Mean no. of ingested SRBC per phagocytic cell.

§ 0.5×10^8 myxospores inoculated per mouse.

|| Significantly different from the control ($P < 0.05$).

Table 2. *DTH response to SRBC in mice inoculated with M. xanthus myxospores*

Treatment (route)	Foodpad swelling* (0.1 mm units)
Saline (i.p.)	6.8 ± 0.9
Myxospores (i.p.)†	2.8 ± 1.9 (<i>P</i> = 0.01)
Saline (s.c.)	7.2 ± 1.2
Myxospores (s.c.)†	6.7 ± 3.5 (NS)

* Difference in footpad thickness (arithmetic means of results from at least six mice ± 95% confidence limits; *P* values obtained by the *t* test). NS, Not significant.

† 0.3×10^8 myxospores inoculated per mouse.

moderate but significant inflammatory response was found 3 d after inoculation of myxospores. Adherent cells did not show significant changes in the treated animals, but the phagocyte fraction of adherent cells was significantly increased 3 d after inoculation. The number of SRBC ingested per phagocytic cell was significantly increased both 1 d and 3 d after myxospore inoculation.

Bone marrow cellularity in mice after inoculation of M. xanthus myxospores. The possible effect of i.p. injection of myxospores on bone marrow cell populations was evaluated 24 h after injection. The counts of nucleated cells per femur were $8.7 \pm 1.53 \times 10^6$ for control mice injected with saline, and $9.0 \pm 1.85 \times 10^6$ for mice injected with 0.5×10^8 myxospores (values are arithmetic means of at least six mice ± 95% confidence limits). These results indicated that myxospores did not alter the number of bone marrow nucleated cells.

Effect of M. xanthus myxospores on the DTH response to SRBC. The results in Table 2 indicate that myxospores suppressed the DTH response when they were inoculated i.p. but not when they were inoculated s.c.

DISCUSSION

The results presented here show that the inoculation of laboratory animals with myxospores of *M. xanthus* causes modulation of the humoral and cellular immune responses against unrelated antigens.

Immunomodulation induced by bacteria provides a useful experimental system in immunobiological studies (Schwab, 1977). A bacterial cell represents a complex association of antigens, mitogens and adjuvants, which may act on several levels of the immune system (Schwab, 1977; Johnson, 1981). In the case of myxospores, it is difficult to attribute their immunomodulation properties to a defined component. In Gram-negative bacteria the lipopolysaccharide (LPS) is recognized as a major fraction with adjuvant properties (Rosenstreich & Mergenhagen, 1975; Koenig & Hoffman, 1979). However, the modulation pattern of the antibody-forming cell response in mice by *M. xanthus* myxospores was quite different from that reported for the LPS from Gram-negative bacteria (Diamantstein *et al.*, 1976; Nakano *et al.*, 1976; Covert & Zarkower, 1971). Furthermore, no LPS has been described among the components of myxospore cells (Sutherland, 1976). LPS and LPS-containing cells have been recognized as potent inducers of rapid inflammatory responses (Snyderman & Vergheze, 1980) and bone marrow cell depletion in mice (Behling & Nowotny, 1981). This was not observed in our assays with *M. xanthus* myxospores. It seems reasonable to assume that bacterial cell fraction(s) other than LPS may have been responsible for the immunomodulation by myxospores in our studies.

Our results support several hypotheses which may explain the mechanisms of immunomodulation by *M. xanthus* myxospores. It appears that the regulatory function of T-suppressor (T_s) cells may be very important in these mechanisms. Thus, suppression of the DTH response in mice after myxospore inoculation may be attributable to T_s cell stimulation, as reported for other

bacterial adjuvants (Galleli *et al.*, 1981). In the same way, suppression of both the IgG primary response and the secondary response to SRBC in rabbits may be explained in terms of T_s-cell stimulation, since it may be comparable to the partial conversion to a T-cell-dependent antibody response in T-independent responses; there is some evidence that limitation of IgG production in T-cell-independent responses is due to active participation of T-cells (Schuler *et al.*, 1982; McKearn *et al.*, 1982). We conclude that *M. xanthus* myxospores provide an interesting inducer of immunomodulation, in which the active bacterial cell material appears to differ from that in Gram-negative bacteria. Further work is required for a better understanding of the immunomodulation mechanisms of this system.

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