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Studies of the immunological activities of the outer membrane protein from *Escherichia coli*

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Summary. The outer membrane protein (OMP) prepared from *Escherichia coli* was found to be a potent mitogen for murine B cells and to be capable of inducing polyclonal antibody formation as well as a proliferative response. Spleen cells from nude mice responded equally as well to OMP as those from their normal litter-mates, whereas nylon-wool-purified T cells or thymocytes failed to respond. The proliferative response was dependent on the presence of macrophages. The macrophage dependency of the polyclonal antibody response seemed to be less than that of the proliferation. OMP was mitogenic for lipopolysaccharide (LPS)-resistant C3H/HeJ spleen cells, further indicating that OMP is an unique B-cell mitogen distinct from LPS.

OMP also enhanced the specific antibody response sixty-seven-fold to an optimal dose of sheep red blood cells (SRBC) *in vitro*. The kinetics of the response, however, was not altered from that of cultures without OMP. The anti-SRBC response of spleen cells from C3H/HeJ mice was also enhanced by the addition of OMP, suggesting that the adjuvant effects were not due to the LPS in the preparation. Antibody responses *in vitro* to TI-1 antigens, trinitrophenyl-LPS (Boivin) (TNP-LPS^{B}) and TNP-*Brucella abortus*, were not enhanced in the presence of OMP. In contrast OMP enhanced the response to TI-2 antigens, TNP-LPS^{W} (Westphal) and dinitrophenyl-Ficoll and T cells were shown to be required for these augmented antibody responses. Enhancement was not seen in nude mouse spleen cell cultures but was seen when nylon-wool-purified T cells were added to the cultures.

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INTRODUCTION

It is well known that the lipopolysaccharide (LPS) has the capacity to initiate non-specifically the proliferation of murine B cells and to induce polyclonal antibody formation. Recently, several reports have provided evidence that, in addition to LPS, other bacterial cell wall components share the capacity to activate B cells. Lipoprotein (LP) from outer membrane of *Escherichia coli* has been demonstrated to be a potent immunostimulant of murine B cells as measured by both proliferation and non-specific antibody formation (Melcher, Braun & Galanos, 1975). The activity of LP seems to reside in the lipid moiety (Bessler & Ottenbreit, 1977). The endotoxin protein (EP) associated with lipid A has also been shown to have B-cell stimulatory property distinct from LPS (Sultzer & Goodman, 1976). It was said, however, that EP was still heterogeneous electrophoretically and at present it is difficult to define the active component (Goodman & Sultzer, 1979). More recently, it has also

been demonstrated that another set of major proteins of *E. coli* outer membrane exhibits similar activities in mice (Bessler & Henning, 1979).

In our laboratory, a protein fraction extracted from *E. coli* was found to show potent inhibitory activity against several tumours in mice. Evidence was provided showing that the activity was not due to LPS in the preparation (Watanabe, 1969; Namiki, Igarashi & Watanabe, 1978). In these reports we have suggested that the outer membrane proteins play a significant role in the anti-tumour activity by enhancing the host response against the tumour. In the present study, one of the major outer membrane proteins (OMP) was isolated by the methods developed by Nakamura & Mizushima (1976) and examined for its immunological activities. The results showed that the protein is a unique mitogen for murine B cells and a polyclonal B-cell activator, and that it exerts potent adjuvant effects on the antibody responses to SRBC or TI-2 antigens but not to TI-1 antigens.

MATERIALS AND METHODS

Mice

Male and female C57BL/6J, C3H/HeJms, BALB/c nude (nu/nu) mice and their normal litter-mates (nu/+) were supplied by the Animal Breeding Unit of our institute. Male C3H/HeJ mice were purchased from Jackson Laboratories, Bar Harbor, Maine, U.S.A. All mice were used at 6–16 weeks of age.

Mitogens and antigens

Outer membrane protein (OMP) was prepared from *E. coli* K-12 YA21 kindly provided by Dr S. Mizushima (Department of Agricultural Chemistry, Nagoya University) according to the methods described by Nakamura & Mizushima (1976). Briefly, *E. coli* cells were disrupted by sonic oscillation and the cell envelope was separated by centrifugation, treated with ribonuclease and deoxyribonuclease, and extracted with Triton X-100 to remove the cytoplasmic membrane. The Triton-treated envelope was further digested with lysozyme and then extracted with sodium deoxycholate. The extract was chromatographed on a Sephadex G-200 column and the peak containing OMP was collected and concentrated. Each step of purification was monitored by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the system described by Mizuno & Kageyama (1978). The final product was purified near to homogeneity. The OMP could be identified with O-10 protein on the basis of two characteristics: heat modifiability and trypsin sensitivity confirmed by the changes in the electrophoretic profile of the heat- or trypsin-treated material. The term O-10 was given by Nakamura & Mizushima (1976) to the outer membrane protein with the tenth biggest molecular weight in the proteins of *E. coli* K-12 YA21. The Limulus test, kindly performed by Dr H. Goto (Department of Internal Medicine of our Institute), revealed that contaminating LPS, if any, was less than 0·01% by weight of the protein. Protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Before addition to cultures, OMP was extensively dialysed against carbonate buffer pH 8·5. LPS from *E. coli* O55 was prepared by the phenol-water methods of Westphal, Lüderitz & Bister (1952). Phytohaemagglutinin-P (PHA) was purchased from Difco Laboratories, Detroit, Mich., U.S.A. It was dissolved in sterile water and used at a final dose of 0·25 μl/culture. Dextran sulphate (DxS) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. LPS^W (LPS prepared by the phenol-water extraction methods of Westphal) and LPS^B (LPS prepared by the Boivin trichloroacetic acid procedures) were purchased from Difco Laboratories, Detroit, Mich., U.S.A. and conjugated with trinitrophenyl (TNP) as described by Jacobs & Morrison (1975). TNP-*Brucella abortus* (TNP-BA) was a gift from Dr J. Mizuguchi, National Institute of Health, Japan. The original suspension of BA contained 10^{11} bacteria/ml. Dinitrophenylalanylglycylglycyl-Ficoll (DAGG-Ficoll) was prepared according to the methods of Inman (1975). The optimum doses of these antigens to induce specific antibody responses *in vitro* were determined in preliminary experiments: TNP-LPS^B, 50 ng/ml; TNP-LPS^W, 50 ng/ml; TNP-BA, 1:50 dilution, 50 μl/ml; DAGG-Ficoll, 50 ng/ml.

Preparation of T cells

Spleen cells were enriched with T cells by passage through a nylon-wool column according to the methods of Julius, Simpson & Herzenberg (1973). Residual B cells were enumerated by the membrane immunofluorescent technique described by Kawamura (1969).

Depletion of adherent cells

Spleen cells were filtered through a Sephadex G-10 column to remove adherent cells (Ly & Mishell, 1974).

The number of residual macrophages was monitored by the uptake of neutral red (Arnaiz-Villena, Gyöngyössy & Playfair, 1974) and by the macrophage-dependent polyclonal antibody response to DxS (Persson, Hämmerström & Smith, 1977).

Lymphocyte culture

Cells were cultured in flat-bottom Microtest II plate (Falcon No. 3042) in a humidified atmosphere of 5% CO_2 in air at 37°. For studying the cell proliferation, $0{\cdot}5 \times 10^6$ cells were cultured in 0·2 ml medium RPMI 1640 with 2% foetal calf serum (FCS) and kanamycin (100 μg/ml) for 2 days, pulsed with 0·25 μCi [^{3}H]-thymidine ([^{3}H]-TdR) per well and then harvested 6–18 hr later with a cell harvester and counted in a liquid scintillation counter. For studying the polyclonal and specific antibody responses, 1×10^6 cells were cultured in 0·2 ml medium RPMI 1640 with 20% foetal calf serum 5×10^{-5} M 2-mercaptoethanol and kanamycin (100 μg/ml) for 3 and 4 days, respectively.

Plaque-forming-cell assay

For studying the polyclonal and specific antibody responses, cells from triplicate cultures were pooled and assayed for plaque-forming cells (PFC) by the technique of Plotz, Talal & Asofsky (1968) with modifications. Specific anti-TNP and polyclonal antibody responses were assayed using TNP-horse red blood cells (TNP-HRBC). TNP-HRBC were prepared according to the methods of Rittenberg & Pratt (1969).

Estimation of adjuvant effects

Since OMP induced polyclonal antibody formation at doses used to assay for the adjuvant activities, the activities were estimated by comparing $\Delta PFC_{antigen+OMP}$ with $\Delta PFC_{antigen}$. They were calculated according to the following formula.
$\Delta PFC_{antigen+OMP} = PFC_{antigen+OMP} - PFC_{OMP}$;
$\Delta PFC_{antigen} = PFC_{antigen} - PFC_{background}$.

RESULTS

Dose-response and kinetics of the OMP-induced mitogenic and polyclonal antibody response

Murine spleen cells were cultured with various concentrations of OMP for 2 days and assayed for [^{3}H]-TdR incorporation, demonstrating that OMP was highly mitogenic towards murine spleen cells. Stimulation of [^{3}H]-TdR incorporation started at a concentration of 0·5 μg/ml OMP and peaked between 50 and 500 μg/ml. In order to study the effect of OMP on B cells, spleen cells were cultured with various doses of OMP for 3 days and assayed for anti-TNP PFC, since cellular pathways leading to B-cell mitogenesis and to polyclonal antibody formation have generally been considered to be closely linked together as was suggested for LPS (Goodman & Sultzer, 1977). OMP elicited a PFC response significantly at concentrations between 5 and 50 μg/ml. In subsequent experiments, 50 μg/ml OMP was used to study both [^{3}H]-TdR incorporation and polyclonal antibody responses. Next, the kinetics of the OMP-induced mitogenic and polyclonal antibody responses were examined. [^{3}H]-TdR incorporation and polyclonal antibody formation reached their peak responses on day 2 and 3, respectively. These results are shown in Fig. 1. Subsequently, therefore, cultures were assayed on days 2 and 3 for [^{3}H]-TdR incorporation and polyclonal B-cell activation, respectively.

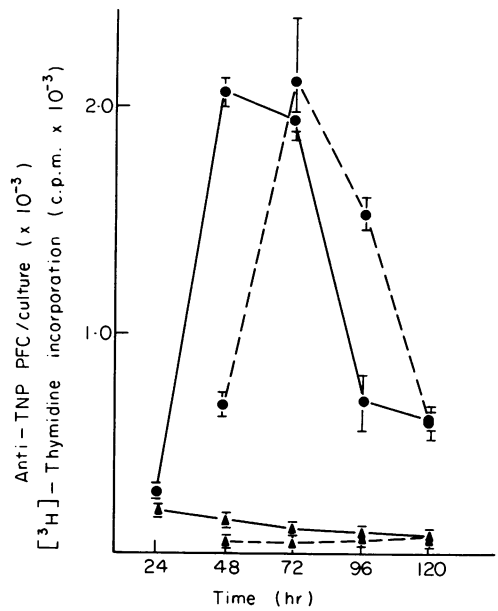

Figure 1. Kinetics of OMP-induced mitogenic and polyclonal antibody responses. Spleen cells were cultured for various intervals shown on the abscissa with or without OMP. (●) 50 μg/ml OMP; (▲) without OMP; (- - -) anti-TNP PFC/culture ($\times 10^{-3}$); (——) [^{3}H]-thymidine incorporation (c.p.m. $\times 10^{-4}$).

Table 1. Mitogenic and polyclonal antibody responses of nude mouse spleen cells to OMP*

	[^{3}H]-TdR incorporation† Mean c.p.m. ± SD/culture		Anti-TNP PFC‡ Mean PFC ± SD/culture	
Stimulator	nu/+	nu/nu	nu/+	nu/nu
None	2369 ± 289	2191 ± 117	65 ± 10	58 ± 15
OMP (50 μg/ml)	21,327 ± 2220	22,680 ± 627	1837 ± 176	1540 ± 142

* Spleen cells from nude mice and their normal litter-mates were cultured with OMP and assayed for [^{3}H]-TdR incorporation and anti-TNP PFC.
† Assayed on day 2 after initiation of culture.
‡ Assayed on day 3 after initiation of culture.

Mitogenic and polyclonal responses of spleen cells from nude mice

In order to study the cell population responsive to OMP, nude mouse spleen cells were cultured with 50 μg/ml OMP and assayed for [^{3}H]-TdR incorporation and anti-TNP PFC. Spleen cells from nude mice used in these experiments showed neither a mitogenic response to PHA nor an antibody response to SRBC, a T-dependent antigen (data not shown), indicating the absence of T-cell function. As shown in Table 1, the OMP-induced mitogenic and polyclonal antibody responses of nude mouse spleen cells were comparable to those of cells from their normal litter-mates, suggesting that the lymphocytes stimulated by OMP were B cells and that OMP can induce proliferation and polyclonal antibody formation in the absence of T cells.

Failure of nylon-wool-purified T cells or thymocytes to proliferate in response to OMP

In order to delineate further the cell population responsive to OMP, nylon-wool-purified T cells or thymocytes were cultured with OMP and assayed for proliferative response. The results are shown in Table 2. The cells non-adherent to nylon wool were enriched with T cells and responded well to PHA but poorly to LPS. The cell population also responded poorly to OMP. Thymocytes did not respond to OMP. Thus, OMP seemed to stimulate B cells exclusively.

Effect of macrophage depletion on the proliferative response of spleen cells

Macrophages have been shown to be implicated as accessory cells for the responses to some mitogens

Table 2. Mitogenic responses of nylon-wool-purified T cells or thymocytes to OMP

	[^{3}H]-TdR incorporation Mean c.p.m. ± SD/culture		
Stimulator	Spleen cells	Nylon T-cells*	Thymocytes
None	601 ± 98	97 ± 6	92 ± 9
LPS (50 μg/ml)	14,430 ± 514	571 ± 55	132 ± 7
PHA	4624 ± 940	14,438 ± 2485	191 ± 13
OMP (50 μg/ml)	13,170 ± 1048	454 ± 60	172 ± 110

* Spleen cells passed through a nylon-wool column.

Table 3. Mitogenic and polyclonal responses of Sephadex G-10 passed cells*

	[^{3}H]-thymidine incorporation Mean c.p.m. ± SD/culture		Mean anti-TNP PFC ± SD/culture	
Stimulator	Untreated	G-10 passed	Untreated	G-10 passed
None	4208 ± 478	357 ± 50	30 ± 5	12 ± 6
LPS (50 μg/ml)	32,458 ± 1903	11,119 ± 1230	818 ± 77	537 ± 51
DxS (50 μg/ml)	13,316 ± 345	754 ± 20	255 ± 15	25 ± 17
OMP (50 μg/ml)	31,541 ± 1479	3,458 ± 259	1280 ± 106	727 ± 133

* Spleen cells were cultured with OMP before and after macrophages were removed by passage through a Sephadex G-10 column.

such as DxS (Persson *et al.*, 1977). To determine the role of macrophages in OMP-induced proliferation and polyclonal antibody formation, spleen cells were cultured with OMP before and after passage through a Sephadex G-10 column. the results are shown in Table 3. The responsiveness of G-10 passed cells was significantly lower than that of untreated spleen cells both in terms of proliferation ($P < 0{\cdot}01$) and polyclonal antibody formation ($P < 0{\cdot}01$), although the responses were never abolished completely as was the response to DxS. The proliferative response (89% decrease) was affected more severely than the polyclonal antibody response (43% decrease) by the macrophage depletion. The results would suggest that both the proliferative and polyclonal antobody responses to OMP are partially dependent on macrophages.

Mitogenic response of spleen cells from C3H/HeJ mice

Since LPS is associated with the outer membrane protein and is a potent mitogen for B cells, it was conceivable that the mitogenic activity of OMP might be due, at least partially, to the contaminating LPS in the OMP preparation. Therefore, the activity of OMP was tested using spleen cells from C3H/HeJ mice which are low responders to LPS (Watson & Riblet, 1974). As shown in Table 4, OMP could act as a mitogen equally well for both C3H/HeJms, normal responder to LPS, and C3H/HeJ cells. This would suggest that the mitogenic activity of OMP is not due to LPS possibly contaminating the preparation.

Table 4. Mitogenic response of C3H/HeJms and C3H/HeJ spleen cells

	[^{3}H]-thymidine incorporation Mean c.p.m. ± SD/culture	
Stimulator	C3H/HeJms	C3H/HeJ
None	2438 ± 253	725 ± 85
LPS (50 μg/ml)	36,717 ± 791	3903 ± 415
OMP (50 μg/ml)	29,483 ± 1307	22,725 ± 1103

Adjuvanticity of OMP

The adjuvant effect of OMP on anti-SRBC antibody response *in vitro* was studied. Since adjuvant effect is often dependent on antigen dose (Morgan, Walker, Thoman & Weigle, 1980), various doses of SRBC ranging from 10^3 to 10^7 were added to the spleen cell cultures and incubated with a constant amount of OMP (5 μg/ml). Anti-SRBC direct PFC were enumerated after 4 days incubation.

As shown in Fig. 2, antibody responses to all doses of SRBC except for the lowest dose (10^3 SRBC per culture) were enhanced by the addition of OMP. Even the response to the optimum dose of SRBC (10^6 per culture) was enhanced more than fifteen-fold. Therefore, the optimum dose of SRBC was used in the following experiments.

Dose of OMP needed for the enhancement of anti-SRBC response and the kinetics of the enhanced response

In order to determine the optimal amount of OMP needed to enhance the anti-SRBC response, various amounts of OMP (5×10^{-2}–5×10^2 μg/ml) were added to the cultures along with 10^6 SRBC and anti-SRBC PFC were assayed on day 4. Cultures were also set up

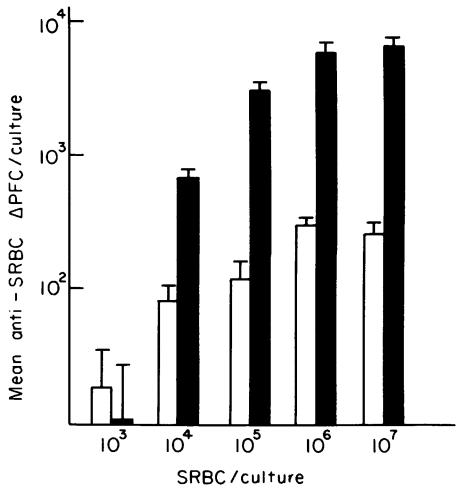

Figure 2. Adjuvant effects of OMP on anti-SRBC response. Spleen cells (1×10^6) were cultured with 10^3–10^7 SRBC/culture and 5 μg/ml OMP for 4 days. PFC was calculated from the following formula as described in Materials and Methods: (□) $\Delta PFC_{SRBC} = PFC_{SRBC} - PFC_{background}$; (■) $\Delta PFC_{SRBC+OMP} = PFC_{SRBC+OMP} - PFC_{OMP}$; $PFC_{background}$, 18 ± 8/culture; PFC_{OMP}, 337 ± 28. The results show the mean ± SD in triplicate cultures.

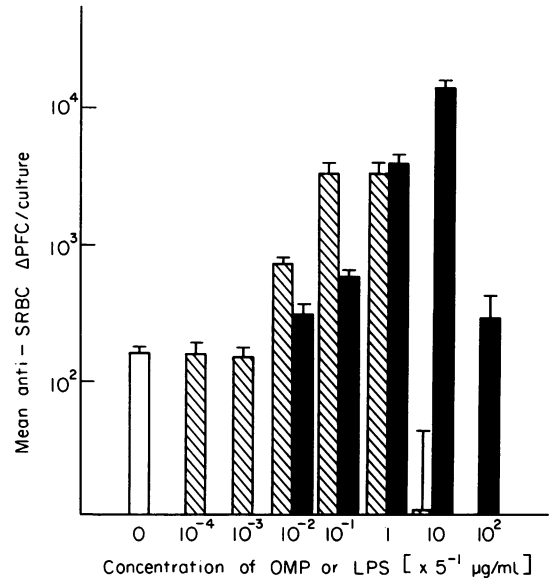

Figure 3. Adjuvant effects of various doses of OMP and LPS on anti-SRBC response. $5 \times 10^{-2} - 5 \times 10^2$ μg/ml OMP (■) and $5 \times 10^{-4} - 5 \times 10$ μg/ml LPS (▨) were added to cultures containing 1×10^6 SRBC and incubated for 4 days. (□) Cultures with SRBC alone. $PFC_{background}$, 13 ± 6/culture; PFC_{OMP}, 47 ± 10 (5×10^{-2} μg/ml), 145 ± 22 (5×10^{-1} μg/ml), 343 ± 20 (5 μg/ml), 440 ± 44 (50 μg/ml), 128 ± 26 (500 μg/ml); PFC_{LPS}, 38 ± 13 (5×10^{-4} μg/ml, 57 ± 18 (5×10^{-3} μg/ml), 82 ± 28 (5×10^{-2} μg/ml), $100 + 30$ (5×10^{-1} μg/ml), 465 ± 66 (5 μg/ml), 192 ± 46 (50 μg/ml).

to which various amounts of LPS (5×10^{-4}–5×10 μg/ml), instead of OMP, were added along with SRBC (10^6/culture). As can be seen in Fig. 3, OMP showed a potent adjuvant effect at all doses tested (note that the ordinate of Fig. 3 is in log scale). The maximal enhancement (sixty-seven-fold) was observed when 50 μg/ml of OMP was added. Higher doses were less active. In contrast LPS-induced enhancement was maximal (thirteen-fold) at a dose of 0·5–5 μg/ml. If OMP should be contaminated with LPS, the amount of LPS possibly present in the preparation was calculated on the basis of the results of the Limulus test mentioned above (less than 0·01% of protein) to be less than 5×10^{-3} μg/ml, which by itself was incapable of exerting any substantial adjuvant effect (Fig. 3). If the adjuvant effect of OMP was due to LPS in the preparation, the effect should be promoted when more than 50 μg/ml of OMP was added to culture, since LPS exerted the effect when cultures contained $0{\cdot}5 \times 10^{-2}$–5 μg/ml LPS (Fig. 3). The results of the experiments, however, do not support this. Anti-SRBC responses of the cultures with 500 μg/ml of OMP were scarcely enhanced (Fig. 3). Thus, the pronounced adjuvant effect of OMP observed cannot be attributed solely to LPS.

In order to study the characteristics of the enhanced response kinetic experiments were conducted. Spleen cells were cultured with SRBC in the presence of 50 μg/ml of OMP, and the responses to SRBC were assayed over a 3–5 day period. The kinetics of anti-SRBC responses were parallel in cultures with and without OMP, reaching the peak on day 4 (data not shown).

Effect of OMP added at various intervals after cultures on the response to SRBC

In order to study the mode of adjuvant activity of OMP, OMP (50 μg/ml) was added at various intervals after the initiation of the cultures with SRBC. As shown in Fig. 4, the maximal enhancement of anti-SRBC response occurred when OMP was added simultaneously with SRBC. The adjuvant effect decreased to approximately 30% when OMP was added at 24 hr and to about 10% when added at 48 hr after

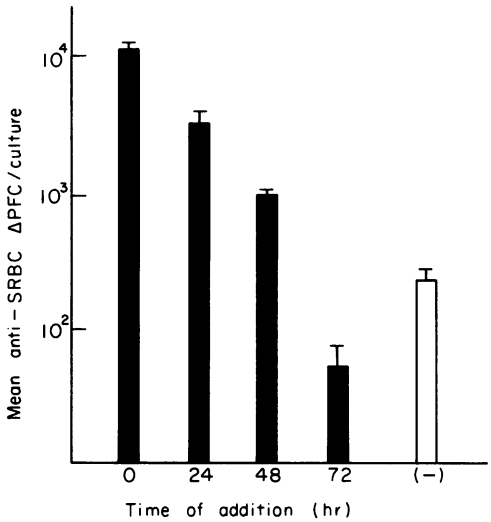

Figure 4. Effect of time of addition of 50 μg/ml OMP to cultures. Spleen cells were cultured with SRBC and OMP added at various times shown on the abscissa. (■) Cultures with antigen and OMP; (□) cultures with antigen alone. $PFC_{background}$: 35±10/culture; PFC_{OMP}: 360±41 (0 hr), 625±140 (24 hr), 225±53 (48 hr), 72±18 (72 hr).

initiation of the cultures. The response was not enhanced when the addition of OMP was delayed by 72 hr. These results indicate that OMP exerts the effect on early events in antibody response.

Adjuvant effect of OMP on the response to T-independent antigens

In order to assess the role of macrophages and T cells in the adjuvant effect of OMP, C57BL/6 spleen cells depleted of macrophages or BALB/c nu/nu mouse spleen cells were cultured with SRBC alone or together with OMP and assayed for anti-SRBC PFC on day 4. Any significant enhancement of response over that of polyclonal activation was not observed in these cultures, suggesting that OMP could not substitute for macrophages or T cells in the anti-SRBC response (data not shown). Then, experiments were performed to see whether OMP could enhance the responses to T-independent antigens. Spleen cell cultures containing the optimal doses of TNP-BA, TNP-LPS^B, TNP-LPS^W, or DAGG-Ficoll were incubated in the presence or absence of OMP for 4 days and assayed for anti-TNP PFC. As shown in Table 5, the antibody responses to TI-1 antigens, TNP-BA and TNP-LPS^B

Table 5. Enhancing effects on responses to T-independent antigens

Antigens*	OMP (50 μg/ml)	Mean anti-TNP ΔPFC±SD/culture†
TNP-LPS^B	−	418±41
	+	617±338
TNP-BA	−	841±78
	+	553±243
TNP-LPS^W	−	331±70
	+	2017±413
DAGG−Ficoll	−	245±78
	+	1747±430

* These antigens were used at their optimal doses determined in preliminary experiments. TNP-LPS^B, 50 ng/ml; TNP-BA 1:50 dilution, 50 μl/ml; TNP-LPS^W, 50 ng/ml; DAGG-Ficoll, 50 ng/ml.

† $PFC_{background}$, 82±21; PFC_{OMP}, 1200±87.

(Fidler, Morgan & Weigle, 1980), were not enhanced by OMP, but the responses to TI-2 antigens, TNP-LPS^W and DAGG-Ficoll, were significantly enhanced.

T-cell requirement for OMP-induced enhancement of antibody responses to TI-2 antigens

As OMP was capable of enhancing the antibody responses to TI-2 antigens and the antigens were reported to be T-dependent in the strict sense of the words (Mond, Mongini, Sieckman & Paul, 1980), it was of interest to know whether T cells were required for the enhancement observed. Spleen cells from nude

Table 6. Adjuvant effects of OMP in nude mouse responses to TI-2 antigens

Antigens	OMP (50 μg/ml)	Mean anti-TNP ΔPFC±SD/culture nu/+	Mean anti-TNP ΔPFC±SD/culture nu/nu†
TNP-LPS^W	−	118±53	130±41
	+	1720±852	685±264
DAGG-Ficoll	−	198±57	70±35
	+	2580±495	185±166

* The responses in BALB/c(nu/+) mice: $PFC_{background}$, 102±8; PFC_{OMP}, 1,585±146.

† The responses in BALB/c (nu/nu) mice: $PFC_{background}$, 57±16; PFC_{OMP}, 1585±146.

Table 7. Requirement for T cells in adjuvant effect of OMP on response to DAGG-Ficoll

Cells*		Mean anti-TNP ΔPFC ± SD/culture§	
B† (%)	T‡ (%)	DAGG-Ficoll	DAGG-Ficoll + OMP
100	0	185 ± 25	258 ± 346
70	30	172 ± 66	1012 ± 320
50	50	178 ± 51	1565 ± 475

* Total number of cells was kept constant (10^6/culture).

† Spleen cells from BALB/c nude mice.

‡ Nylon wool non-adherent cells.

§ $PFC_{background}$, 65 ± 15 (B:T = 100:0), 80 ± 5 (B:T = 70:30), 117 ± 6 (B:T = 50:50); PFC_{OMP}, 1375 ± 331 (B:T = 100:0), 1330 ± 262 (B:T = 70:30), 1410 ± 74 (B:T = 50:50).

The response in nu/+ mice: $PFC_{background}$, 47 ± 10; PFC_{OMP}, 1510 ± 62; $PFC_{DAGG\text{-}Ficoll}$, 203 ± 56; $PFC_{DAGG\text{-}Ficoll+OMP}$, 3240 ± 317.

mice and their normal litter-mates (nu/+) were cultured with these antigens in the presence or absence of OMP and assayed for anti-TNP PFC on day 4. The responses of (nu/+) cells to these antigens were enhanced markedly as in the preceding experiments, whereas the response of (nu/nu) cells was moderately enhanced (TNP-LPS^W) or not enhanced (DAGG-Ficoll) (Table 6). This would suggest that some T cell participation was needed to implement the adjuvant effect of OMP on response to these antigens.

In order to confirm the requirement for T cells increasing numbers of nylon-wool-purified spleen cells were added to spleen cells from nude mice keeping the total cell number constant (1×10^6/culture), and the anti-TNP responses to DAGG-Ficoll were measured after 4 days culture. As shown in Table 7, the OMP-induced potentiation was resumed by the addition of T cells in a dose-dependent manner and fully restored in cultures containing 50% T cells and 50% nude mouse spleen cells.

DISCUSSION

In the present paper, we have shown that OMP is a potent mitogen for murine B cells and induces polyclonal antibody formation, and also exerts striking adjuvant effects on antibody responses. Several observations suggest that OMP is active in its own right and that its properties are not due to LPS contaminating the preparation. First, the mitogenic activity of OMP (Table 4) and its adjuvant effects (data not shown) were equally well manifested with spleen cells from LPS-resistant C3H/HeJ mice as with those from normal mice. Secondly, the amount of LPS in the OMP preparation was estimated to be less than 0·01% of the protein and the potent adjuvant effects of OMP could not be explained by such minute amounts of LPS. Furthermore, taking advantage of the fact that OMP is sensitive to trypsin (Nakamura & Mizushima 1976), OMP was treated with insoluble trypsin and assayed for the adjuvant effect after removal of trypsin. It was found that the adjuvant effect of OMP was reduced by the trypsin treatment to about one-seventh of the untreated material (data not shown), suggesting again that the active principle of OMP is protein and not LPS. On the bassis of these findings, it is reasonable to consider that the activities of OMP are distinct from those of LPS, although the possibility cannot be excluded that small amounts of LPS have some effect(s) on OMP activities.

Bessler & Henning (1979) reported that protein I isolated from the outer membrane of *E. coli* by their own methods was a potent mitogen and polyclonal activator in mice, while protein II* was only weakly active. OMP used in the present experiments corresponds to protein II* according to their nomenclature (DiRienzo, Nakamura & Inoue, 1978). Our results, therefore, are not in accord with those of Bessler & Henning (1979). It is, at present, difficult to delineate the reason responsible for the different results.

In the proliferative response to OMP, spleen cells from nude mice generated a comparable response to that of normal cells, indicating that the responding cells were B cells and that T cells were not required for the response. Nylon-wool-purified T cells as well as thymocytes failed to give rise to a proliferative response, lending further support to the above notion. The polyclonal response was also independent of T cells as indicated by the experiments with nude mouse spleen cells.

The mitogenic and polyclonal antibody responses to OMP were significantly reduced but could not be abrogated by the depletion of macrophages. These results indicated that spleen cell responses to OMP were partially dependent on macrophages. The partial dependence of the OMP response on macrophages could be explained by postulating the involvement of two separate subsets of B cells, one responding directly to OMP while another responding indirectly

through interaction with macrophages. The polyclonal antibody response to OMP was less dependent on macrophages. Then, it follows that the former B-cell population would preferentially differentiate into antibody-forming cells in response to OMP.

OMP also has striking effects on specific antibody responses. The IgM anti-SRBC PFC response was increased much more dramatically by OMP than by LPS at their optimum doses of 50 and 5 μg/ml, respectively (sixty-seven-fold versus thirteen-fold). Characteristically, pronounced enhancements of the anti-SRBC response occurred over a wide range of antigen dose from 10^4 to 10^7 SRBC/culture. In view of the fact that some other bacterial adjuvants are effective only when suboptimal dose of antigen is employed, it may be noteworthy that the response even to the optimal dose of antigen was markedly augmented by OMP. The adjuvant effects of LPS (Sjöberg, Anderson & Möller, 1972) and synthetic muramyl dipeptide (Specter, Cinprich, Friedman & Chedid, 1978) on anti-SRBC responses occur most dramatically only when a low number of SRBC are added to culture. Also, the Fc fragment-induced adjuvant effect on anti-SRBC response is most pronounced when suboptimal amounts of antigens are employed (Morgan *et al.*, 1980). By contrast, enhancement of responses to TNP-SRBC and TNP-Ficoll by anti-IgD antibody requires high antigen dose (Finkelman, Woods, Wilburn, Mond, Stein, Berning & Scher, 1980). In the latter case, it was hypothesized that large quantities of antigen are needed for production of a high level of T-cell help or for optimum focusing of helper factors on to B cells.

For a full expression of adjuvant effect of OMP *in vitro*, OMP must be present for the entire culture period. The fact that OMP is required early in the response for the enhancement to occur would favour the concept that OMP-induced proliferation of primed B cells, which precedes differentiation, plays a significant role in the enhancement. It is also conceivable that as discussed for the Fc fragment-induced adjuvanticity (Morgan *et al.*, 1980), OMP requires a period of time for the generation of an effective signal which must be given at 24–60 hr of culture in order to enhance the helper T-cell function.

When OMP was added together with SRBC to the culture of spleen cells depleted of macrophages or T cells, no enhancement of anti-SRBC response over that of polyclonal activation was seen (data not shown), indicating that OMP cannot replace the function of T cells or macrophages. Thus, OMP differs from LPS in that the latter can overcome the requirement for helper T cells in antibody response to T-dependent antigens (Sjöberg *et al.*, 1972). The effects of OMP on the responses to TI antigens were examined. While no substantial increase in the response to TI-1 antigens, TNP-LPS^B and TNP-BA, was seen, the responses to TI-2 antigens, TNP-LPS^W and DAGG-Ficoll, were significantly enhanced. Moreover, it has been shown by the experiments with nude mice that the enhancement is T-cell-dependent. The T-dependence of the response was further confirmed by the reconstitution experiments. The T-cell involvement in the responses to TI antigens seems at first sight paradoxical. However, it was reported recently that the anti-IgD-induced enhancement of the response to TNP-Ficoll appears to be T-cell-dependent (Finkelman *et al.*, 1980). It is suggested that an auxillary help provided by T cells is required for the optimal response to TNP-Ficoll (Mond *et al.*, 1980). It may well be that OMP works in some way to augment such T-cell help resulting in an enhanced response to TNP-Ficoll.

The OMP enhancement of the response to T-dependent antigen, SRBC, is much more pronounced compared with that to TNP-Ficoll, and no substantial augmentation of the responses to TI-1 antigens was observed. These differences in the adjuvant effects of OMP may also be explained in terms of the extent of the T-cell dependence of the responses to these antigens. The responses to TI-1 antigens seems to be much less dependent on T cells than the responses to TI-2 antigens (Mond *et al.*, 1980). If OMP acts mainly through increasing the T-cell help, the adjuvant effects will depend in turn on the amount of such help required for the response to these antigens.

Thus, OMP could be considered to modulate the defence mechanisms of the host through exerting mitogenic and polyclonal activation of B cells to stimulate non-specific antibody formation, also through exerting adjuvant effects on T cells to stimulate specific antibody formation.

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