Brief Definitive Reports

IMMUNOLOGICAL ADJUVANTS AND THE MECHANISM OF CELL COOPERATION

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Immunological adjuvants are defined as agents which act nonspecifically to enhance immune responsiveness to a specific antigen. While many substances are known to have adjuvanticity, remarkably little is understood about the mechanism by which they enhance either the antibody response or cellular immunity. It is now clear, for at least some antigens, that interaction of three cell types is required for antibody formation: thymus-derived lymphocytes (T cells), bone marrow-derived lymphocytes (B cells), and probably macrophages. In studies on the specificity of response to hapten-protein conjugates, it has emerged that the specificity of the antibody-producing cell (hapten specific) is generally not the same as that of the T cell which bears immunological memory (carrier specific) (1–3). In addition, the characteristics of the helper cell involved in antibody formation, i.e. thymus derived, nonantibody secreting, carrier specific, and nondividing, appear at present indistinguishable from those of the thymus-derived lymphocyte responsible for cell-mediated immunity (4).

We chose to pursue this analogy by exploring the effects of the two most effective immunological adjuvants in mice, pertussis vaccine and tubercle bacilli, the latter at least, known also to engender delayed-type hypersensitivity. The in vitro immunization system developed by Mishell and Dutton (5) permitted us to study the effect of adjuvant on a primary in vitro response to sheep erythrocytes (SRBC).

We have posed the following questions: (*a*) Will spleen cells from animals primed with adjuvant give an enhanced in vitro primary response against an irrelevant antigen, SRBC? (*b*) If so, what is the mechanism by which the adjuvant-primed cells enhance this in vitro response? (*c*) Which cell is responsible for the adjuvant effect and upon what cell does this cell act?

Materials and Methods

The adjuvants used in this study were *Bordetella pertussis* vaccine, fluid, USP (E. Lilly & Co., Indianapolis, Ind.) containing 8 antigenic units (AU/ml (equivalent to no fewer than 6.4×10^{10} standard pertussis organisms/ml). For use in vitro, the organisms were dialyzed

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against saline or washed by centrifugation to remove the preservative. The tubercle bacilli were of two types, seemingly not significantly different in their action. BCG, Phipps strain (TMC 1029), and *Mycobacterium tuberculosis*, R1RV (TMC 205), a human avirulent organism, were generously provided by the Trudeau Institute, Saranac Lake, N. Y., and grown in Dubos albumin-Tween medium to a density of approximately 5×10^8–2×10^9 viable organisms/cc. The BCG were killed by heating, and one batch of R1Rv with a count of no greater than 5×10^3 was used. Purified protein derivative was obtained from the Ministry of Agriculture, Central Veterinary Laboratories, Weybridge, England.

$B6D_2F_1$ (C57BL/6 × DBA/2) mice were immunized once with intraperitoneal (0.4 AU) and subcutaneous inoculation (0.4 AU) in two flanks of *B. pertussis* or the killed equivalent of 4–10 × 10^7 tubercle bacilli. Mice were used between 3 and 5 wk after immunization.

Spleen cultures were prepared in the manner described by Mishell and Dutton (5) enriched in Eagle's minimal essential medium and 7% fetal calf serum (Reheis Co., Inc., Berkley Heights, N. J.; lot No. 21806). The cells were cultured at 7×10^6 cells/cc in Falcon plastic Petri dishes (Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles, Calif.; catalogue 3001) to which were added 25 μl of a 2% SRBC suspension, and gassed with a mixture containing 7% O_2, 10% CO_2, 83% nitrogen. All cultures were fed daily with nutrient medium, and the numbers of direct plaque-forming cells (PFC) was assayed on days 3 or 5 by the Jerne plaque assay in 0.5% agarose. The results are expressed as plaque-forming cells per 10^6 recovered cells, as determined by an appropriately calibrated Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.). Dilutions of pertussis vaccine or PPD were added usually on day 0 to adjuvant-primed and normal spleen cultures. All cultures were made in duplicate, and each sample was plated at two dilutions, each in duplicate. Thus an average of four or eight plates was counted for each sample.

RESULTS AND CONCLUSIONS

When adjuvant-primed cells were cultured with SRBC, a normal primary in vitro response ensued (Table I). However, when specific adjuvant to which the donors were primed was added to the cultures, an enhanced response was obtained, which is described by an adjuvant index (A.I.), i.e., the ratio of the response to SRBC in the presence of adjuvant compared with the response of the same cells with no adjuvant added in vitro. The maximal adjuvant effect of a 2–3-fold stimulation in the culture system described was obtained on the 3rd day. While the number of PFC was greatest on the 5th day, the adjuvant effect was less marked. The degree of enhancement was affected by the dose of adjuvant added to the cultures; higher doses of adjuvant were often found to be toxic. The enhancement produced by adjuvants added in vitro was dependent on specific prior priming of the donor spleens, rather than on any nonspecific effect on lymphoid cells, since the adjuvants did not enhance significantly the response to SRBC of unprimed cells. Lastly, the adjuvant effects were specific in the sense that cultures of primed cells were significantly stimulated only by the adjuvant in vitro related to that used for priming, although slight cross-stimulation by PPD is seen on pertussis cells, which might perhaps be expected on the basis of common endotoxin activity.

In cell-mediated immunity it has been shown that soluble mediators are produced by sensitized lymphocytes upon interaction with antigen in vitro (4). Since the enhanced response of adjuvant-primed spleens depended upon challenge in vitro with specific adjuvants, analogous to the production of

mediators in delayed-type hypersensitivity, experiments were designed to ascertain whether adjuvant-primed cells produced a soluble mediator which could enhance the response of normal cells to SRBC. Spleen cells from adjuvant-primed mice were cultured as described above with antigen, *B. pertussis*, at 10^{-4} AU/ml or PPD at 25 μg/ml. At 20 hr, the cells were removed and the supernatant harvested. These were then added in three dilutions to normal spleen cells together with sheep erythrocytes and cultured for 3 days. As can be seen in Table II, supernatants of adjuvant-primed cells stimulated in vitro with the specific adjuvant did enhance the primary response of normal cells to SRBC. Moreover, when the response produced by the supernatants of pertussis-primed spleens was compared with that produced by the control super-

TABLE I

Primary In Vitro Response to SRBC of Adjuvant-Primed and Normal Spleen Cells

Priming of donor	Adjuvant in vitro		Day 3 response	A.I.‡	Day 5 response	A.I.‡
			*PFC/10^6 cells** ± SE		*PFC/10^6 cells** ± SE	
Pertussis	None	—	20.3 ± 1.2 (11)	1.00	669 ± 101 (7)	1.00
	Pertussis	1.0–0.2 × 10^{-4} AU	40.1 ± 2.9 (11)	1.97	298 ± 34 (7)	0.45
		4.0–0.8 × 10^{-5} AU	75.5 ± 3.7 (5)	3.72	824 ± 142 (5)	1.23
	PPD	25 μg	33.4 ± 2.5 (7)	1.64		
Tubercle bacilli	None	—	53.8 ± 8 (5)	1.00	500 ± 65 (8)	1.00
	PPD	25 μg	129 ± 20 (5)	2.40	454 ± 38 (8)	0.91
		5 μg	86.0 ± 9 (5)	1.60	617 ± 91 (4)	1.22
	Pertussis	1.0–2.2 × 10^{-5} AU	36.7 ± 3.7 (4)	0.68		
None	None	—	30.1 ± 2.4 (7)	1.00	879 ± 153 (4)	1.00
	Pertussis	1.0–0.2 × 10^{-4} AU	30.1 ± 2.0 (7)	1.00	336 ± 71 (4)	0.38
		4.0–0.8 × 10^{-5} AU	45.5 ± 5.5 (3)	1.52	788 ± 197 (3)	0.89
	None	—	36.0 ± 9.1 (3)	1.00	631 ± 74 (5)	1.00
	PPD	25 μg	46.0 ± 10 (3)	1.28	582 ± 72 (5)	0.92
		5 μg	40.3 ± 12 (3)	1.11	662 ± 109 (4)	1.05

* Numbers in parentheses indicate the number of experiments.

‡ A.I. adjuvant index.

natants of normal spleen cultures cultured with pertussis, a dose-response relationship was observed in the adjuvant index. In our experience, the adjuvant effect of pertussis-primed spleens was greater and more reproducible than that for PPD-stimulated tubercle bacilli-primed spleens. In the case of pertussis, a given supernatant stimulated strongly about two of three normal spleens on which it was tested, while, for a given normal spleen, the optimal amount of active supernatant required to produce maximal enhancement similarly varied from spleen to spleen. It appeared in the case of pertussis that the degree of adjuvanticity for normal spleens of the active supernatants was of the same order as, or only slightly less effective than that of adjuvant-primed spleens.

Lastly, we have tried to ascertain the cell type responsible for producing this supernatant factor and that upon which it acts by means of a mouse anti-θ isoantiserum. The AKR anti-θ C3H serum was prepared by the method

of Cerottini et al. (6). Spleen cells were incubated at 37°C for 45 min at a final concentration of 10^7 cells/ml containing anti-θ serum and rabbit complement, both at 1/18. The average per cent cytotoxicity in four experiments was approximately 27%. Pertussis-primed cells were treated with anti-θ serum or normal AKR mouse serum in parallel, and cultured with pertussis. At 20 hr supernatants were assayed for adjuvanticity (Table III A). In these experiments it appeared that the anti-θ treatment completely abrogated the adjuvant effect, i.e. production of supernatants that enhanced the primary response of normal spleens, while normal mouse serum had no effect. In an additional four experi-

TABLE II

Enhancement of In Vitro Response to SRBC of Normal Spleen Cells By Supernatants of Adjuvant-Primed Spleen Cells

Vol. supern.	Supern. of pertussis-primed cells cultured with pertussis*	Supern. of normal cells cultured with pertussis*	A.I.§
m	*PFC/10^6 cells ± SE*		
0.2	166 ± 17	73 ± 8	2.27
0.1	109 ± 8	62 ± 7	1.76
0.05	100 ± 14	136 ± 36	0.76
0	73 ± 7	73 ± 7	1.00
	Supern. of Tbc.-primed cells cultured with PPD‡	Supern. of normal cells cultured with PPD‡	
0.2	111 ± 7	120 ± 45	0.92
0.1	105 ± 8	121 ± 16	0.86
0.05	217 ± 16	125 ± 36	1.73
0	105 ± 12	121 ± 13	0.87

* 4 supernatants were examined in 10 trials, excepting at 0.05 ml where 3 were tested in 6 trials.

‡ 4 supernatants were examined in 6 trials, excepting at 0.05 ml where 2 were tested in 3 trials.

§ Adjuvant index, in this case, is given by PFC (supern. of primed cells + adjuvant)/PFC (supern. of normal cells + adjuvant).

ments, active supernatants from pertussis-primed spleens were added to cultures of normal cells which had been pretreated either with anti-θ or normal AKR mouse sera (Table III B). The data indicate that the supernatant factor was capable of exerting an adjuvant effect equally well on spleens deficient of T cells. These results suggest that it is the T cell which carries specific information for adjuvant priming and which produces the soluble factor which mediates the effect. Moreover, they suggest that this mediator affects either B cells directly or macrophages, rather than T cells in the recipient population, although clearly all T cell function was not abolished in controls after anti-θ treatment. The studies described confirm the observations of Allison and Davies (7) that the adjuvants studied here in the mouse, pertussis and tubercle bacilli, require the participation of thymus-derived lymphocytes for their effect. To-

gether with the work of Dutton et al. (8) they demonstrate that the Mishell-Dutton in vitro system provides a remarkable tool for studying the effects of immunological priming with related, unrelated antigens and adjuvants. They confirm and extend the findings of Rubin and Coons (9) which indicated that spleen cells of mice primed to tetanus toxoid show enhanced primary in vitro responses to SRBC on day 5 when the specific protein is added to the culture on day 2. Perhaps because of the antigenic complexity of the pertussis, the degree of enhancement here is two to five times greater. They further support the finding that T cells can produce a soluble factor(s) after mixed leukocyte cultures (8), or from incubation with antigen (10–12) which can replace T cells in a primary in vitro response.

TABLE III

Effect of Anti-θ Serum on Production and Action of Enhancing Supernatants

	A.I.*	
	Primed cells treated with	
A. Effect on cells producing supernatants	NMS‡	Anti-θ
Supernatants from normal cells cultured with pertussis (0.2 ml)	1.00 (control)	1.09 ± 0.04
Supernatants from pertussis-primed cells cultured with pertussis (0.2 ml)	2.30 ± .22	1.02 ± 0.11
	Normal cells treated with	
B. Effect on normal cells exposed to enhancing supernatants	NMS	Anti-θ
Supernatants from normal cells cultured with pertussis (0.2 ml)	1.00 (control)	0.59 ± 0.08
Supernatants from pertussis-primed cells cultured with pertussis (0.2 ml)	3.37 ± 0.81	3.36 ± 0.90

* A.I., adjuvant index = PFC (experimental culture)/PFC (control culture).

‡ NMS, normal mouse serum.

While it may be tempting to speculate about the role of such a soluble mediator in vivo, the system is really too complex to allow simple interpretation. It must be recognized that the rocking cultures of the in vitro immunization system bear little resemblance to the tight architecture of a lymphoid organ where diffusion of molecules from cell to cell must be quite restricted. Further, it is probable that a variety of factors are produced, as in the case of cell-mediated immunity, of which some may be stimulatory and others toxic. This might explain why a greater effect was seen at day 3 rather than day 5.

These observations indicate that the effects of immunological adjuvants can be studied effectively in vitro, and that some adjuvants may not indeed act so nonspecifically as previously believed (13), but rather by virtue of an immunologically specific T cell response to antigenic portions of these adjuvants. They further support the possibility that the T cell in this system, like that in

cell-mediated immunity, when stimulated by the specific antigen can act by producing a soluble mediator(s) which affects B cells or perhaps macrophages. They do not, however, imply that the soluble mediator is the sole mechanism by which T cells can exert a cooperative effect on B cells.

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