ACTIVATION OF T AND B LYMPHOCYTES IN VITRO

II. Biological and Biochemical Properties of an Allogeneic Effect Factor (AEF) Active in Triggering Specific B Lymphocytes*

By DIETER ARMERDING‡ and DAVID H. KATZ

(From the Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115)

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Delineation of the complex events involved in requisite cooperative interactions between thymus-derived (T) and bone marrow-derived (B) lymphocytes in the development of antibody responses to all but a selected class of antigens has been the subject of intensive analysis in many laboratories (reviewed in reference 1). A major effort in this regard has been focused on the identification of various biologically active substances capable of influencing lymphocyte function on antibody responses to different antigens elicited in in vitro systems. Such studies have utilized either a variety of mitogenic substances originating from sources other than lymphocytes (reviewed in reference 2) or substances generated during lymphocyte activation, and presumably lymphocyte derived, in the course of in vitro cell responses to either mitogens, antigens, or both (3–21).

The demonstration by Dutton et al. (3) that supernatants obtained from short-term cultures of histoincompatible mouse spleen cells contained a nonantigen-specific biologically active mediator capable of markedly affecting in vitro antibody responses to thymus-dependent antigens provided evidence for the existence of such a possible mediator participating in T-B-cell cooperation. In the succeeding 3 yr, these basic findings have been confirmed and extended in several ways. First, it has been also shown that other means of lymphocyte activation, such as exposure to specific antigen or nonspecific mitogens, also generate nonantigen-specific biologically active mediators capable of variably influencing B- and/or T-cell responses in vitro (4–21). Second, there have been reports that another type of biologically active lymphocyte factor(s) which has antigen specificity can be generated and recovered from culture supernatants in certain systems (9, 22–26). It is not yet clear whether two distinct active moieties, one nonspecific and the other antigen specific, participate together or in selected circumstances in the development of antibody responses.

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The present experiments have focused on the biological and biochemical properties of a nonspecific mediator produced by populations of activated T lymphocytes during short-term in vitro reactions with foreign alloantigens. We have analyzed the activity of the unseparated and of chromatographically separated fractions of the supernatants from such cultures on the in vitro responses of mouse lymphocytes to soluble 2,4-dinitrophenyl (DNP)-carrier conjugates and also to heterologous erythrocytes. The results demonstrate that a highly active protein moiety in the mol wt range of 30,000–40,000 is capable of acting directly on B lymphocytes, in the presence of antigen, to effect triggering and subsequent differentiation and proliferation to antibody-forming cells in vitro. The active molecule(s), although not manifesting specificity for antigen, does exhibit some strain-specific properties suggesting a possible relationship to antigens or other gene products coded in the major histocompatibility gene complex.

Materials and Methods

The proteins, hapten-carrier conjugates, and SRBC antigens were prepared and used in a manner identical to those described in our previous reports (27–29).

Animals and Immunizations.—Mice of the inbred lines DBA/2, C3H, BALB/c, C57BL/6, and (DBA/2 × C3H)F_1 hybrids (C3D2 F_1) were obtained from the Jackson Laboratories, Bar Harbor, Maine. Mice were immunized at 2–3 mo of age intraperitoneally with 100 μg of DNP-protein conjugates emulsified in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and were used as primed spleen cell donors 2–6 mo thereafter. Mice used for spleen cell donors in primary in vitro responses were 2–5 mo of age. Thymus cell donors for the preparation of activated T cells were 6–8 wk of age, the recipients 10- to 12-wk old.

Allogeneic Supernatants.—Preparation of allogeneic supernatants were induced in the following way (14–16, 30): DBA/2 thymus cells were activated against C3H histocompatibility antigens by injecting irradiated DBA/2 recipients (600 R) intravenously with 1×10^7 thymus cells from 5- to 8-wk old DBA/2 mice mixed with 1×10^7 irradiated (2,000 R) C3D2F_1 spleen cells. The spleens of these recipient animals were removed after 8 days. The recovery of (activated) T cells was about 20% of the original inoculum. 1×10^7 activated T cells were cultured together with 1×10^7 irradiated (2,000 R) C3D2F_1 spleen cells in 1 ml of culture medium (31, 32). Supernatants were harvested 24 h later. It should be noted that this procedure virtually eliminates potential contributions from B lymphocytes or their specific antibodies to the supernatant. Pilot experiments performed to establish optimal conditions for induction of the most active supernatants in this way showed that: (*a*) fetal calf serum (10%) was required in the medium; otherwise, supernatants were either frequently quite suppressive or only low in enhancing activity; and (*b*) culture periods exceeding 24 h markedly increased the amounts of inhibitory materials in the supernatants. Thus, all supernatants employed in the following experiments were derived from 24-h cultures with fetal calf serum-supplemented medium. These supernatants are referred to also as allogeneic effect factors (AEF)[1] in this publication. All allogeneic supernatants of identical cultures were

[1]*Abbreviations used in this paper*: AEF, allogeneic effect factors, ASC, extract of *Ascaris suum*; BGG, bovine gamma globulin; BSA, bovine serum albumin; D-GL, copolymer of D-glutamic acid, D-lysine; $DNP_{2.1}$-ASC, moles of DNP × 10^{-7} per mg of ASC protein; DNP_{20}-FγG, moles of DNP per mole of FγG; DNP_{14}-KLH per 100,000 mol wt units of KLH; DNP_8-OVA, moles of DNP per mole of OVA; PγG, fowl gamma globulin; KLH, keyhole limpet hemocyanin; MLC, mixed lymphocyte cultures; OVA, ovalbumin; PBS, 0.01 M phosphate-buffered saline.

pooled, centrifuged for 15 min at 1,500 *g* to remove the cells, and filtered through a 0.45 μg Millipore filter (Millipore Corp., Bedford, Mass.). They were then distributed into small aliquots and kept frozen at −20°C.

Purification and Chromatography of the AEF.—

Sephadex gel chromatography: 1.5–2.0 ml of 10-fold-concentrated allogeneic supernatant (UM-2 Ultrafiltration Membranes, Amicon Corp., Lexington, Mass.) or, for control, of 10% fetal calf serum-containing medium were applied to a Sephadex G-200 column (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) (2.5 × 90 cm) which was equilibrated with phosphate-buffered saline (PBS). All chromatography procedures were done at 4°C and under semisterile conditions using sterile endotoxin-free solutions of PBS for prewashing the gel and for elution at a flow rate of 11 ml/h. Individual fractions were pooled as indicated in Results (Fig. 4) and concentrated to the original volume with, according to the molecular weight range, the appropriate ultrafiltration membranes (UM-2 or PM-10). They were sterilized by filtration through 0.45 μg Millipore filters and kept frozen.

Pooled and concentrated biologically active fractions were rechromatographed on a Sephadex G100 column (2.5 × 90 cm) in PBS at a flow rate of 18 ml/h. The column was calibrated using bovine gamma globulin (BGG) (mol wt, 150,000), bovine serum albumin (BSA) (mol wt, 65,000), and bovine pancreatic insulin (mol wt, 36,000 in associated form under salt and pH conditions employed) as markers. Fractions were concentrated to the original volume.

Heat treatment: Sensitivity of the AEF against heat was tested using the complete supernatant and incubating 1-ml aliquots in a 56°C waterbath for 1 h.

Trypsin digestion: Digestion was performed using the complete allogeneic supernatant and 10% of thoroughly washed agarose-coupled trypsin (Enzite Agarose Trypsin, Miles-Servac, England) for 10 h at 37°C. The supernatant was used afterwards as a 1:5 dilution for cell culture.

Depletion of T Lymphocytes.—The preparation of anti-θ-serum, determination of anti-θ-serum cytotoxicity, and anti-θ-serum treatment of spleen cells have been previously described (33).

Spleen Cell Cultures and Determination of In Vitro Antibody Responses.—The Mishell-Dutton system (31, 32) was used with modification for culturing cells in microcultures described previously (27). Briefly, cultures were set up in a total volume of 0.3 ml in three steps antigen, AEF (or plain medium in appropriate controls), and cells were added to the culture wells in this order. Cell harvesting and assays for anti-DNP and anti-SRBC plaque-forming cells (PFC) were performed as previously described (27).

Preparation of Antigen-Bound Macrophages.—Induction and harvesting of peritoneal macrophages from unprimed mice were carried out as detailed in previous reports (34, 35). Uptake of antigen was performed by exposing 2.5×10^6 macrophages to 4 μg of DNP-protein (in a total volume of 1.4 ml) for 30 min on ice; the cells were then washed thoroughly and resuspended in medium at a concentration of 2.5×10^5 macrophages/ml. Under these conditions the percent uptake of DNP-protein ranged from 0.25 to 0.35 (35). 100 μl of these cells (2.5×10^4 cells) were added to each culture well as the only source of antigen. Macrophages incubated without antigen served as background controls. The optimal dose of macrophages of 2.5×10^4/culture was established in pilot experiments. This number of macrophages contained about 0.125–0.175 μg of DNP-protein. The cell density of the spleen cells in these cultures was 1.67×10^6 cells/ml.

RESULTS

Reconstitution of Helper Cell Function in Primary In Vitro Anti-SRBC Antibody Responses by AEF.—Fig. 1 demonstrates the strong activity of AEF generated in supernatants from 24 h one-way MLC reactions between DBA/2 activated T cells and heavily irradiated C3D2F$_1$ target spleen cells on the pri-

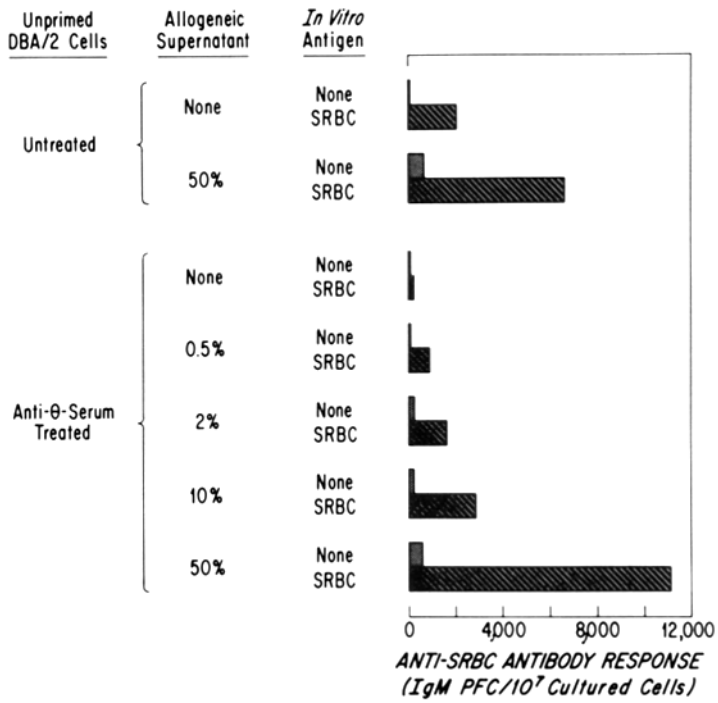

FIG. 1. Generation of allogeneic supernatant activity from 24 h one-way MLC reactions between DBA/2-activated T cells and heavily irradiated C3D2F$_1$ target spleen cells. Untreated and anti-θ-serum-treated spleen cells from unprimed DBA/2 mice were cultured without or with SRBC in the presence of various concentrations of supernatant as indicated. The primary IgM anti-SRBC antibody responses after 4 days are shown.

mary in vitro IgM anti-SRBC antibody response of either untreated or anti-θ-serum-treated DBA/2 spleen cells. The following results are remarkable: (*a*) AEF enhanced the antigen-independent background as well as the antigen-dependent response of untreated spleen cells; (*b*) The AEF reconstituted the helper cell function of the T-cell-depleted cell population proportionately to the concentration of supernatant added; (*c*) The increase in PFC response from 6,630 for the untreated cells to 11,080 for the T-cell-depleted cultures corresponds to the enrichment of B cells by the anti-θ-serum treatment (killing rate was about 45%). The IgG anti-SRBC response (not shown) paralleled the IgM response.

Reconstitution of Helper Cell Function in Secondary In Vitro Anti-DNP Antibody Responses by AEF.—The effects of AEF on secondary IgG anti-DNP responses to DNP-keyhole limpet hemocyanin (KLH) of untreated and anti-θ-serum treated spleen cells from DNP-KLH-primed DBA/2 donors are shown in Fig. 2. Effects on IgM responses (not shown) were parallel. The addition of AEF resulted in moderate enhancement of the secondary anti-DNP response

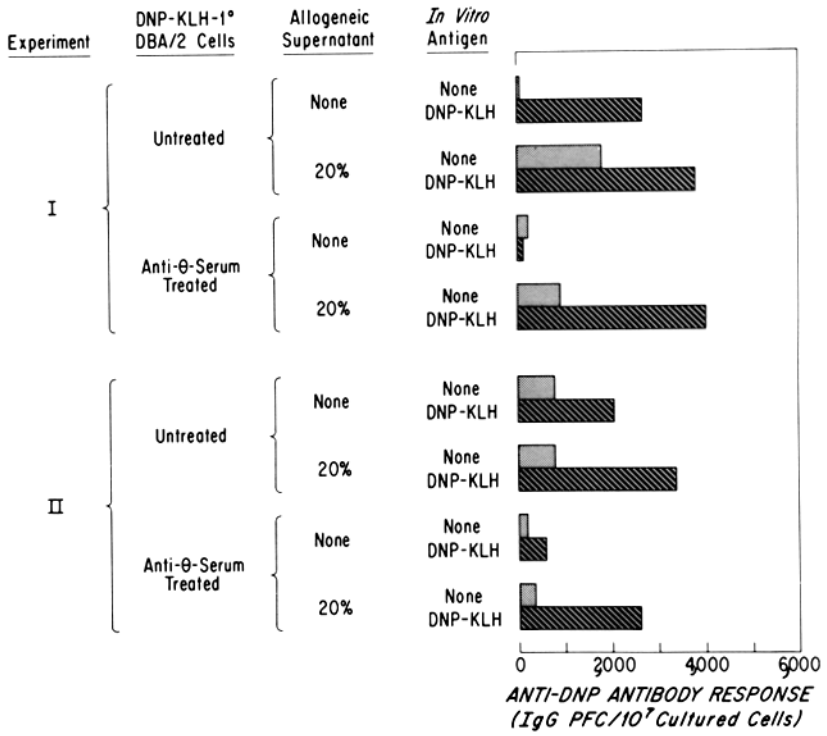

FIG. 2. Reconstitution of helper cell function in secondary in vitro anti-DNP antibody responses by allogeneic effect factors. Untreated and anti-θ-serum-treated spleen cells from DNP-KLH-primed (4 mo) DBA/2 mice were cultured (cell density 3.33×10^6 cells/ml) with or without DNP-KLH (1 μg/ml) in the presence or absence of 20% allogeneic supernatant as indicated. The IgG anti-DNP antibody responses after 4 days are shown.

of untreated cells to DNP-KLH in both experiments. In exp. 1, AEF also induced a considerable increase in the number of background anti-DNP PFC. Again, the addition of AEF reconstituted helper cell function in T-cell-depleted cultures, but no appreciable B-cell enrichment effect was observed.

The Capacity of AEF to Bypass the Requirement for Carrier-Specific Helper T Cells in Secondary In Vitro Anti-DNP Antibody Responses.—The possibility that AEF may actually replace T-cell functions and act directly on B lymphocytes was tested by culturing DNP-KLH-primed DBA/2 spleen cells with or without AEF and with either the homologous conjugate, DNP-KLH, or various heterologous conjugates (DNP-ovalbumin[OVA], DNP-*Ascaris*[ASC], DNP-fowl gamma globulin[FγG], and DNP-D-glutamic acid, D-lysine[D-GL]) as soluble in vitro antigens. The data (IgG responses) from four such experiments are summarized in Table I. These experiments point out the variability of results obtained in this system in certain conditions. In the absence of AEF, the consistent observations in all such cultures was the strong secondary response to

TABLE I

*Effect of AEF on Secondary In Vitro Anti-DNP PFC Responses to Heterologous DNP-Carrier Conjugates**

Exp.	In vitro antigen	IgG anti-DNP antibody response	
		No AEF	AEF (20%)
1	None	67	320
	DNP-KLH	1,950	2,610
	DNP-OVA	40	200
	DNP-ASC	0	300
2	None	618	480
	DNP-KLH	3,658	3,927
	DNP-OVA	518	528
	DNP-Fγ G	384	1,386
	DNP-D-GL	568	1,584
3	None	100	334
	DNP-KLH	2,700	2,520
	DNP-OVA	66	1,504
	DNP-Fγ G	34	1,570
4 *a*	None	0	186
	DNP-KLH	1,860	3,000
	DNP-Fγ G	80	213
4 *b*	None + Mϕ‡	53	213
	DNP-KLH-Mϕ bound‡	1,320	1,860
	DNP-Fγ G-Mϕ bound‡	186	2,155

* DNP-KLH primed DBA/2 spleen cells were cultured at a density of 1.67×10^6 cells/ml for 4 days with or without antigen (1 μg/ml), in the presence or absence of AEF (20%). Anti-DNP PFC/10^7 cultured cells are given. Donors were 3.5-4 mo primed.

‡ 2.5×10^4 macrophages with or without antigen bound were added to each culture.

DNP-KLH and the failure of such cells to respond to any of the heterologous conjugates. The addition of AEF to cultures, however, resulted at times in development of secondary responses to heterologous DNP-carriers (cf. DNP-Fγ G and DNP-D-GL in exp. 2, DNP-OVA and DNP-Fγ G in exp. 3), and at times had no demonstrable effect on such responses (cf. DNP-OVA and DNP-ASC in exp. 1, DNP-OVA in exp. 2, and DNP-Fγ G in exp. 4 *a*). The IgM responses paralleled the IgG responses in these experiments.

The variable activity of AEF on responses to heterologous DNP-carrier conjugates might be related to the form in which the specific determinants are presented to the B cell, and it is conceivable that this may vary from one primed cell preparation to another. Such a variable could be the relative amounts of cell-bound vs. free antigenic determinants which exists in any given culture experiment. This possibility is supported by one experiment in Table I

where the addition of AEF failed to stimulate a secondary response to DNP-FγG added to cultures in free solution (exp. 4 *a*) but resulted in an excellent response to macrophage-bound DNP-FγG (Exp. 4 *b*). Note as well the excellent response to macrophage-bound DNP-KLH. A second experiment is summarized in Fig. 3 where, again, the presence of AEF markedly enhanced the secondary response to macrophage-bound as contrasted to free DNP-FγG.

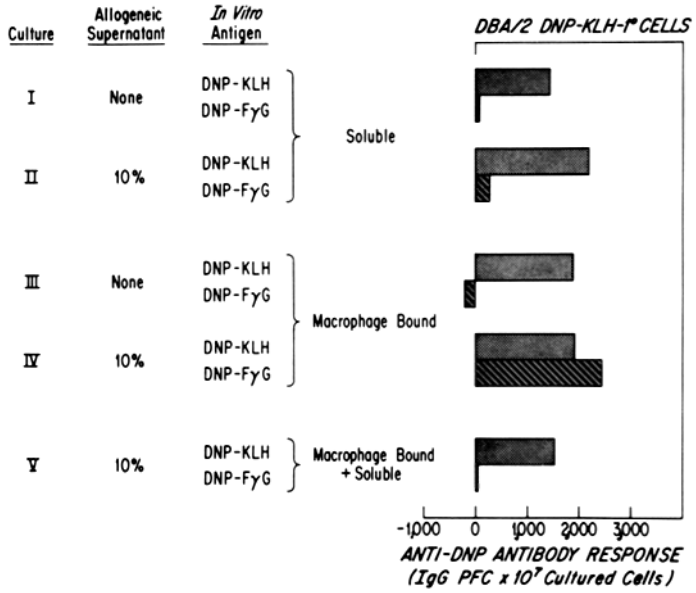

FIG. 3. The capacity of AEF to bypass the requirement for carrier-specific helper T cells in secondary in vitro anti-DNP antibody responses. Spleen cells from DNP-KLH-primed (4 mo) DBA/2 mice were cultured at a cell density of 1.67×10^6 cells/ml with or without antigen in the presence or absence of 10% allogeneic supernatant as indicated. Soluble DNP-KLH or DNP-FγG were added at a concentration of 1 μg/ml (cultures I, II, and V). Macrophage-bound antigen was added on 2.5×10^4 macrophages/well (cultures III, IV, and V). The IgG anti-DNP responses after 4 days are shown. Background values for nonantigen-stimulated control cultures have been subtracted from each corresponding experimental value for purposes of clarity.

More importantly, in view of the possibilities considered above, addition of free soluble antigen to cultures containing macrophage-bound DNP-FγG completely abolished the AEF-enhanced secondary response to DNP-FγG. These data therefore demonstrate, the importance of the structural form in which determinants are presented to B cells in order to observe enhancing activity of AEF.

Partial Biochemical Characterization of the AEF.—In a first series of experiments, crude supernatants were treated in several ways as shown in Table II and then tested for activity in anti-SRBC responses. The results clearly show

TABLE II

*Properties of the AEF: Enhancement of the In Vitro Primary Anti-SRBC Response and Reconstitution of Helper Cell Function with Various Preparations of AEF**

Culture	Treatment of AEF	In vitro antigen	Anti-SRBC antibody response			
			Untreated cells		Anti-θ-serum-treated cells	
			IgM-PFC	IgG-PFC	IgM-PFC	IgG-PFC
1	No AEF	None	0	0	0	0
		SRBC	320	520	40	60
2	Untreated AEF (20%)	None	80	20	680	520
		SRBC	2,560	2,080	3,480	3,040
3	Heated AEF (1 h 56°C)	None	48	33	184	167
		SRBC	1,155	825	600	390
4	Trypsin-digested AEF	None	17	0	0	27
		SRBC	280	100	1,120	200

* Unprimed, untreated, or anti-θ-serum-treated DBA/2 spleen cells were cultured with or without SRBC (1%) for 4 days in the presence or absence of allogeneic supernate (20%) which was previously treated in the manner indicated. Anti-SRBC PFC/10^7 cultured cells are given.

that both activities of AEF, namely, the enhancing effect on the in vitro PFC response of untreated lymphocytes to SRBC and the capacity to reconstitute helper cell function in cultures of anti-θ-serum-treated cells are sensitive to heating for 1 h at 56°C and to digestion with trypsin.

The allogeneic supernatants were next subjected to repeated chromatographic separation on Sephadex G-200 and G-100 and the various eluent fractions tested for activity. The experiments depicted in Figs. 4 and 5 show that the biological activity of the AEF is associated with a molecule of homogeneous shape and size, as evidenced by the fact that the active moiety elutes in a well-defined, albeit small, peak on both gel beds (cf. Fraction V in Fig. 4 and Fraction B in Fig. 5). If the AEF are globular proteins, the mol wt is in the range of 30,000–40,000 since activity coincides most nearly with the position of insulin on G-100 (Fig. 5). Further calibration of this column demonstrated defined peaks for chymotrypsinogen A (mol wt 25,000) and ribonuclease A (mol wt 13,700) separating in subsequent fractions from the gel bed (not shown).

Activity of the AEF on In Vitro Antibody Responses of Cells from Various Mouse Strains.—The biological activity of the AEF requires that it interacts with either: (*a*) the antigen, (*b*) at least one, and perhaps more, of the cells participating in the induction of an antibody response, or (*c*) with both antigen and responding cell(s). This assumption implies that the AEF might have specificity for either the antigen or for one or more of the involved cell types. The following experiments were designed to assess the latter possibility using

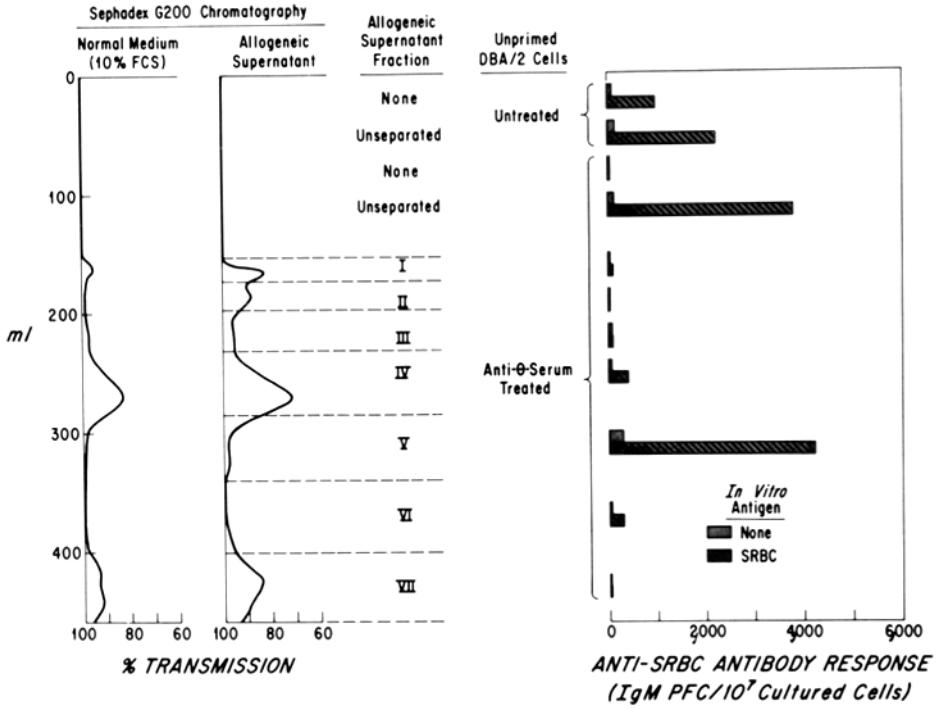

FIG. 4. Comparison of activities of unseparated and Sephadex G-200-fractionated AEF on primary anti-SRBC responses of untreated and anti-θ-serum-treated DBA/2 spleen cells. The chromatography elution patterns of normal culture medium and allogeneic supernatant on Sephadex G-200 are illustrated on the far left. The responses of untreated (top two groups) and anti-θ-serum-treated cells in the presence of equal concentrations (50% in reference to the original unseparated supernatant) of various fractions of the G-200 eluent and a 50% concentration of unseparated supernatant, as indicated, were determined. The IgM anti-SRBC PFC after 4 days in the absence or presence of SRBC are shown.

the crude AEF prepared by reacting DBA/2 activated T cells with target C3D2F_1 cells and testing its activity on the secondary anti-DNP responses of untreated and anti-θ-serum-treated DNP-KLH-primed spleen cells from DBA/2, C3H, (C3D2)F_1, and BALB/c mice. As shown in Table III, AEF almost fully reconstituted helper function in responses of the T-cell-depleted DBA/2 and (C3D2)F_1 populations; in addition, AEF markedly increased background PFC and the secondary response of untreated cells to DNP-KLH in cultures of (C3D2)F_1 cells. The activity of AEF on cells from C3H mice was considerably less, whereas the response of untreated BALB/c cells to DNP-KLH was, in fact, suppressed by the presence of AEF at the concentration employed (20%). This suppressive effect of unpurified AEF was observed over a concentration range down to 2.5% (not shown).

These data indicate either that crude AEF contains an inhibitory moiety

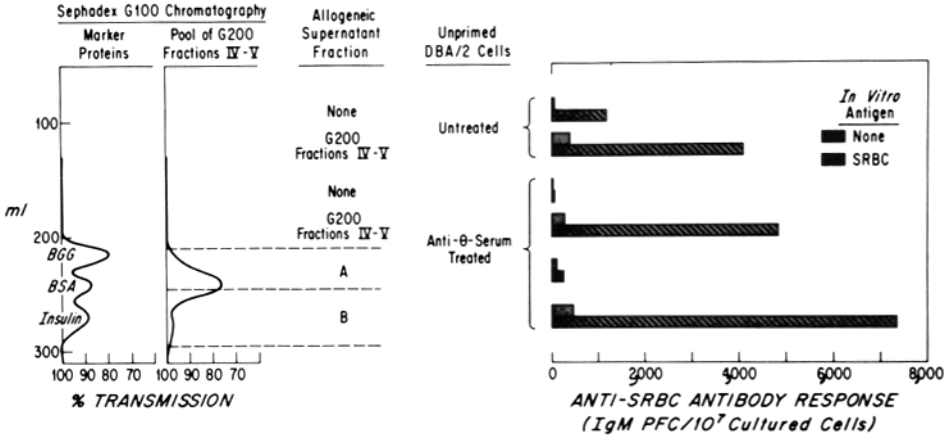

FIG. 5. Fractions IV and V from G-200 (see Fig. 4) were pooled and subjected to further chromatography on G-100. The elution patterns of this supernatant and the corresponding markers BGG (mol wt 150,000), BSA (mol wt 65,000), and insulin (mol wt 36,000 in hexameric form) are shown on the far left. The primary IgM anti-SRBC responses of untreated and anti-θ-serum-treated DBA/2 cells in the presence of the various fractions indicated (50% concentration in reference to the original unseparated supernatant) are shown.

that acts on BALB/c but not on DBA/2 spleen cells, or, alternatively, a mixture of distinct moieties having, respectively, enhancing and inhibitory activities and that the enhancing factor acts best on DBA/2 while the inhibitory factor acts preferentially on BALB/c cells. We attempted to dissect these possibilities by comparing the activities of crude AEF and active G-200 chromatographic fractions of AEF on the primary anti-SRBC responses of spleen cells from DBA/2, C3H, BALB/c, and C57BL/6 mice. As shown in Table IV, unpurified AEF (10%) had the effect of: (*a*) enhancing the response of untreated and reconstituting (with enrichment) the response of anti-θ-treated DBA/2 cells; (*b*) not influencing the response of untreated C3H cells; and (*c*) suppressing the responses of untreated and not reconstituting the responses of anti-θ-treated cells both in BALB/c and C57BL/6 strains. The addition of purified AEF (2%) to cultures of these same cells resulted in marked increases in responses of all strains and, particularly important, was capable of fully reconstituting BALB/c anti-θ-treated cells; the degree of reconstitution with C57BL/6 cells was, in contrast, only partial.

DISCUSSION

The studies presented here have demonstrated the biological and certain biochemical properties of an active moiety produced in supernatants of short-term (24 h) in vitro reactions between T cells specifically activated to foreign alloantigens and the corresponding target cell population. These experiments are based on previous results of in vivo (36, 37) and in vitro (38–40) experimentation in systems of T-B-cell cooperative interactions.

TABLE III

*Reconstitution of Helper Cell Function in Secondary In Vitro Anti-DNP Antibody Responses of Various Mouse Strains with AEF**

Exp.	Strain	Cells	In vitro antigen	IgG anti-DNP response	
				No AEF	AEF (20%)
1	DBA/2	Untreated	None	26	1,440
			DNP-KLH	3,400	3,780
		Anti-θ-serum treated	None	146	68
			DNP-KLH	440	3,000
2	C3H	Untreated	None	203	317
			DNP-KLH	1,000	1,680
		Anti-θ-serum treated	None	160	239
			DNP-KLH	234	510
3	(C3D2)F_1	Untreated	None	200	1,202
			DNP-KLH	2,760	4,500
		Anti-θ-serum treated	None	26	1,320
			DNP-KLH	360	1,920
4	BALB/c	Untreated	None	66	168
			DNP-KLH	5,400	2,400
		Anti-θ-serum treated	None	80	360
			DNP-KLH	360	840

* DNP-KLH-primed, untreated, or anti-θ-serum-treated spleen cells (cell density, 1.67×10^6 cells/ml) from various mouse strains were cultured with or without DNP-KLH (1 μg/ml) in the absence or presence of AEF for 4 days. Figures presented are PFC/10^7 cultured cells. Donors were primed 3 mo (exp. 1), 5 mo (exp. 2) and 3.5 mo (exp. 3 and 4).

One of the questions to which we have addressed our studies is whether an active moiety generated in vitro behaves in a virtually identical biological fashion as the phenomenon we termed the "allogeneic effect" operates in vivo (36, 41). One very pertinent reason why this question should be asked is that, heretofore, studies with allogeneic supernatants have been largely performed on in vitro responses to particulate antigens, such as heterologous erythrocytes or haptenated erythrocytes, in which circumstances positive results have been rather reproducibly obtained (3–5, 9–11, 13–16, 18, 41). On the other hand, in the one previously published study in which the activity of allogeneic cell supernatants on in vitro responses to soluble DNP-carriers was examined, the enhancing effect could only be observed on responses to a thymus-independent DNP-carrier (9).

This apparent distinction between the capacity of allogeneic factors to stimulate B-cell responses to particulate but not soluble antigens has been clarified by the following observations in the present study: The extent to which AEF will either exhibit a reconstituting effect or permit the development

TABLE IV

*Reconstitution of Helper Cell Function in Primary In Vitro Anti-SRBC Antibody Responses of Various Mouse Strains with Unpurified and Sephadex G-200-Purified Allogeneic Effect Factors**

Culture	Strain	Cells	In vitro antigen	IgM anti-SRBC response		
				No AEF	Unpurified AEF (10%)	Purified AEF (2%)
1	DBA/2	Untreated	None	50	184	200
			SRBC	450	1,710	3,420
		Anti-θ-serum treated	None	50	167	351
			SRBC	60	3,930	6,180
2	C3H	Untreated	None	67	67	412
			SRBC	300	340	1,200
		Anti-θ-serum treated	None	40	93	665
			SRBC	40	700	2,060
3	BALB/c	Untreated	None	267	251	251
			SRBC	1,050	570	1,920
		Anti-θ-serum treated	None	13	186	519
			SRBC	60	300	2,730
4	C57BL/6	Untreated	None	468	134	268
			SRBC	1,200	300	2,400
		Anti-θ-serum treated	None	0	106	240
			SRBC	40	60	720

* Normal, untreated, or anti-θ-serum-treated spleen cells from various mouse strains (cell density, 1.67×10^6 cells/ml for C57BL, 3.33×10^6 cells/ml for DBA/2 and BALB/c, and 5×10^6 cells/ml for C3H) were cultured with or without SRBC (1%) in the absence or presence of AEF for 4 days. Figures presented are PFC/10^7 cultured cells.

of secondary anti-DNP responses to heterologous DNP-carriers is clearly determined by the form in which the specific DNP determinants are presented to the B cell. Thus, the presence of AEF failed to stimulate responses of DNP-KLH-primed cells to free soluble DNP-FγG but elicited excellent secondary responses to the same conjugate bound to macrophage surface membranes (Exp. 4, Table I and Fig. 3). Moreover, the response to macrophage-bound DNP-FγG in the presence of AEF was totally abolished by addition of free soluble DNP-FγG to the same culture. It appears, therefore, that the presence of a sufficient quantity of free DNP-carrier molecules might render all or some of the DNP-specific B cells refractory to the action of AEF or, for that matter, the product(s) of carrier-stimulated T cells if the latter are in any way different from AEF. This conclusion is in keeping with earlier observations made in our laboratory in a similar in vitro system (35). It is most probably for this reason that considerable variability was observed in the capacity of AEF to stimulate responses of primed lymphocytes to heterologous DNP-carriers (Table I);

hence, primed spleen cell populations with greater quantities of cytophilic antibodies and/or high affinity B cells may bind relatively more antigen onto cell surfaces capable of triggering other lymphocytes than might occur in a different spleen cell population.

Another point of concern in drawing an analogy between the mechanism of the allogeneic effect in vivo and the activity of AEF and similar factors in vitro is whether or not absolute proof exists as to the direct action of such factors on B cells in vitro. This is not an inconsequential problem in validly interpreting any of these systems since it is possible for a biologically active substance to rapidly expand and/or stimulate differentiation of small numbers of T cells present in a given cell population. A note of caution in this regard was raised previously by others (3, 5, 18). We have tested this possibility in the present studies by analyzing the capacity of AEF to stimulate B cells exposed to the DNP-D-GL copolymer. This compound has been demonstrated to be highly tolerogenic for DNP-specific B cells both in vivo and in vitro under normal circumstances (29, 42–44). However, when administered to appropriately primed animals during a critical time period after induction of an allogeneic effect, DNP-D-GL can provide a definite inductive stimulus for primary or secondary anti-DNP antibody responses (29, 41, 45, 46). Since no demonstrable T-cell function specific for the D-GL carrier has been demonstrated, these observations provided the strongest indirect proof that the allogeneic effect is mediated by a direct interaction on the responding B cells. The capacity of AEF to permit in vitro responses to DNP-D-GL in the present experiments (Table II) and a number of comparable experiments (not shown) constitutes conclusive evidence, therefore, that the active moiety involved is acting directly on B lymphocytes.

The present experiments have demonstrated the following salient physicochemical features of the active enhancing molecule in AEF: (*a*) it is a trypsin-sensitive protein; (*b*) it is labile to heating (56°C, 1 h), thereby indicating the importance of tertiary structure to activity; and (*c*) it has a mol wt in the range of 30,000–40,000. The crude supernatant studied here consists of at least two distinguishable activities, one enhancing and the other inhibitory. Inasmuch as the inhibitory activity can be largely removed by separatory gel chromatography, it is at least possible to state that some of the inhibitory material is higher in molecular weight than the enhancing moiety. On the other hand, it has not yet been definitely established that the active enhancing fraction does not contain inhibitory as well as enhancing activity. Similarly, the existence of inhibitory and/or additional enhancing activities in the various higher molecular weight fractions must be established or ruled out by extensive analyses of the biological activity of these fractions over broad dose ranges.

Thus far, a variety of active supernatants have been described which differ in estimated mol wt from 27,000 to 300,000 (16, 18–20, 47). It is possible that all of these factors are indeed distinctly different physicochemical moieties

which share common biological properties. However, one possibility is that certain of these factors are acting predominantly on T cells and T-cell function and little, or not at all, on B cells. This is virtually ruled out in the present studies by the capacity of AEF to stimulate B-cell responses to DNP-D-GL and in the studies of Feldmann and Basten in which the allogeneic factor enhanced anti-DNP responses of nude spleen cells to the T-cell-independent antigen DNP-flagellin (9). On the other hand, the systems used by Rubin and Coons (8, 12, 19) and Gorczynski et al. (7, 14, 16) leave this possibility open since in the former case the activity of the factor has never been tested on T-cell-deprived spleen populations (8, 12, 19) and in the latter studies the active factor fails to stimulate B cells in the absence of irradiated normal T cells in the same culture (7, 14, 16). Another possible reason for differences may be related to aggregation of lower molecular weight molecules, before chromatographic procedures employed, which could obscure the validity of molecular weight estimates.

The present studies have also questioned whether there may be genetic restrictions governing the biological activity of AEF. Recent observations demonstrating that most effective cooperative T-B-cell interactions occur when the respective cell populations possess identical genes located within the *H-2* gene complex led us to postulate the existence of a membrane "acceptor" site on the B-cell surface that interacts with either a complementary molecule existing either as a T-cell surface site or as a product released from the T cell (48–50). It occurred to us, therefore, that the active enhancing moiety in AEF might indeed represent one such molecule and, if so, should manifest some relationship to histocompatibility specificities. One might predict, for example, that the active enhancing molecule should act best or preferentially on B cells derived from the same histocompatibility type as the effector T lymphocytes producing the molecules.

The studies carried out by us thus far, and presented here, are far from definitive in this regard. However, there is a clear suggestion of the possible preferential activity of AEF on syngeneic B lymphocytes. Thus, AEF from supernatants of mixed cultures containing DBA/2 activated T cells and irradiated $C3D2F_1$ target cells clearly exhibited the greatest enhancing and reconstituting effects on B lymphocytes from DBA/2 and $C3D2F_1$ mice, both of which possess the DBA/2 ($H\text{-}2^d$) histocompatibility molecules (Table III). The purified AEF, although fully reconstituting C3H ($H\text{-}2^k$) and BALB/c ($H\text{-}2^d$) B-cell responses, only partially reconstituted responses of B cells from C57BL/6 mice (Table IV). It is not surprising to observe activity on the cells bearing the C3H haplotype since the target cells bearing such surface antigens might also release active molecules with preferential activity on C3H cells. Moreover, preliminary absorption studies with various spleen cell populations have demonstrated that spleen cells from normal DBA/2 mice are considerably more effective than cells of other strains in absorbing the biological enhancing activity from AEF.

Although a considerable further analysis is essential to define the relationship of the active moiety of AEF to histocompatibility gene products, it is interesting to point out that heretofore unexplained enhancing activity in unstimulated culture supernatants of syngeneic thymocytes or splenic lymphocytes (3, 6, 11, 14) may be explained by this hypothesis. Thus, in certain conditions it would not be surprising to find sufficient shedding, either spontaneously or as a result of nonspecific stimulation in the culture medium, of a histocompatibility gene product normally present on the T-cell surface that might be capable of enhancing and/or reconstituting responses of other B cells. Furthermore, this hypothesis explains the capacity to observe "bystander" effects in mixed culture in vitro since in all such studies demonstrating this phenomenon, the responding bystander B-cell populations have been syngeneic or isologous with the effector T-cell population (3, 15, 38–40).

In summary, as an extension of our previously stated hypothesis on this point (1, 35, 41, 49), we envisage the following sequence involved in effective T-B-cell interactions: The antigen-activated T lymphocytes, in close proximity to the appropriate B cell, either engages direct contact at the specific acceptor site(s) on the B-cell surface and/or releases the active products (AEF) that have specific complementarity for, and bind to, the specific acceptor sites on the B lymphocyte. It is likely that the antigen-binding event on the specific B-cell surface Ig receptors has already taken place before the relevant interaction with the T cell occurs. Perhaps one of the important consequences of the antigen-binding event followed by subsequent movement, capping, and endocytosis of the B-cell surface immunoglobulin receptors (51) is to either appropriately expose greater numbers of acceptor sites or even induce steric conformational changes in the acceptor molecule. This may explain in part why various maneuvers, other than specific antigen binding, leading to Ig receptor movement result in a more highly responsive B-cell population (52). Likewise, it is more conceivable that the process of activation of the T cell results in a critical steric or structural alteration in the active moiety on its cell surface which allows it then to interact with the B-cell acceptor. This mechanism would explain the discriminant regulation involved in normal immune responses since only those T and B lymphocytes whose surface sites have been either appropriately exposed or structurally altered by activation events would be capable of successful interaction. We believe that the respective molecules on T and B cells governing the interaction between these cells are products of a gene or genes in the histocompatibility gene complex. Experiments designed to identify the molecular specificity of AEF and its corresponding binding site on the B cell should resolve this issue.

SUMMARY

The studies presented herein have focused on the biological and biochemical properties of a nonspecific mediator produced by populations of activated T lymphocytes during short-term in vitro reactions with foreign alloantigens. We

have analyzed the activity of the unseparated and of chromatographically separated fractions of the supernatants from such cultures on the in vitro responses of mouse lymphocytes to soluble and macrophage-bound DNP-carrier conjugates and also to particulate heterologous erythrocytes. The results demonstrate that a highly active protein moiety, termed allogeneic effect factors (AEF), in the mol wt range of 30,000–40,000, is capable of acting directly on B lymphocytes, in the presence of antigen, probably to effect triggering and subsequent differentiation and proliferation to antibody-forming cells in vitro. The active molecule, although not manifesting specificity for antigen, does exhibit some strain-specific properties suggesting a possible relationship to cell surface antigens or other gene products coded in the major histocompatibility gene complex.

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