IN VITRO ANALYSIS OF ALLOGENEIC LYMPHOCYTE INTERACTION

I. Characterization and Cellular Origin of an Ia-Positive Helper Factor-Allogeneic Effect Factor*

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The immune response (I) region of the mouse *H-2* major histocompatibility complex regulates interaction between thymus-derived (T) and bone-marrow-derived (B) lymphocytes that result in antibody synthesis (reviewed in references 1 and 2). Immune response (Ir) genes control specific immune responses to various synthetic and native antigens (3) but their cellular site of action (T cells and/or B cells) remains unresolved (reviewed in reference 4). *I*-region compatibility may (5, 6) or may not (2, 7–9) be required for optimal T cell – B cell collaboration during a secondary IgG antibody response. The *I*-region also determines a highly polymorphic series of membrane glycoproteins of approximately 30,000 mol wt denoted as *I*-region-associated (Ia)[1] antigens. These antigens are expressed predominantly on B cells (reviewed in reference 1), may serve as a B-cell differentiation marker (10),[2] may mediate certain steps of the switch from IgM to IgG antibody production. (10, 11), and are purported to be components of antigen-specific helper (12) and suppressor (13) factors as well as an antigen-nonspecific helper (14) factor. Implicit in these results is the involvement of Ia antigens in *Ir* gene function. However, the precise definition of this relationship as demonstrated by the cellular origin of these factors, their target and mechanism of action, and the role of their Ia molecules in lymphocyte interaction is still not yet clear.

Allogeneic effect factor (AEF) has been identified as an antigen-nonspecific, soluble, T-cell-derived, helper factor that mediates in vitro B-cell activation in a manner similar to the in vivo allogeneic effect (14). The active components of this AEF have been reported to be Ia antigens (15) and B_2-microglobulin (16). However, so far it has been difficult to demonstrate directly the presence of Ia antigens on helper T cells (10). In addition, AEF activity seems to reside with proteins of mol wt of 40–45,000 and 12,000 daltons, respec-

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[1] *Abbreviations used in this paper:* AEF, allogeneic effect factor; DNP, 2,4-dinitrophenyl; FCS, fetal calf serum; Ia, I region-associated; Ir, immune response; KLH, keyhole limpet hemocyanin; MLR, mixed lymphocyte culture reaction; PFC, plaque-forming cell; RAMP, rabbit anti-X5563 IgG myeloma protein.

[2] Delovitch, T. L., J. L. Press, and H. O. McDevitt. 1976. Expression of murine Ia antigens during embryonic development. Manuscript in preparation.

tively. This chromatographic profile closely resembles that obtained upon isolation of *H-2* antigens (17).

The experiments described in this report were performed to determine the immunochemical nature and cellular origin of AEF to further elucidate the role of Ia antigens in T cell–B-cell interaction. AEF was produced in an *H-2* congenic strain combination by an initial in vivo allogeneic activation and was harvested from the culture medium of a mixed lymphocyte culture reaction (MLR) between activated responder cells and irradiated stimulator cells. This AEF can provide helper cell function for a primary and secondary antibody response of both responder strain and stimulator strain spleen B cells. Ia antigens derived from both the activated responder and irradiated stimulator cell populations are present in this AEF. By contrast, an AEF generated in the presence of Ia-negative activated responder cells and irradiated T-cell-depleted stimulator cells helps hapten-primed B cells of only the stimulator haplotype. It contains Ia antigens determined only by the stimulator haplotype and not the responder haplotype. The data suggest that this genetically restricted AEF is an Ia-positive helper factor produced by either a B cell and/or macrophage. They also indicate that the haplotype specificity observed is mediated by the interaction of Ia antigens in restricted AEF and Ia antigens on a B cell, which activates hapten-primed B cells to IgG antibody production. Preliminary reports of these data have been previously published (10, 18, 19).

Materials and Methods

Mice. All inbred strains of mice used were maintained in our colony at Stanford University or at University of Toronto.

Antigens. A single batch (batch 03) of sheep erythrocytes (SRBC), previously determined to elicit a high direct plaque-forming cell (PFC) response, was purchased from Grand Island Biological Co., Grand Island, N.Y. Burro erythrocytes were purchased from Woodlyn Laboratories, Guelph, Ontario, Canada. Keyhole limpet hemocyanin (KLH) was supplied by Pacific Biomarine Supply Co., Venice, Calif. Conjugates of 2,4-dinitrophenyl (DNP) DNP_{11}-KLH (per 10^5 daltons) were prepared as previously reported (20).

Immunizations. Mice were injected intraperitoneally with either 5×10^8 SRBC in Dulbecco's phosphate-buffered saline or 100 μg of alum precipitated DNP_{11}-KLH mixed with 2×10^9 heat-killed *Bordetella pertussis* organisms (purchased from Connaught Medical Research Laboratory, Willowdale, Ontario, Canada). They were sacrificed 6–12 wk later and used as primed spleen cell donors.

Antisera. Anti-*H-2* and anti-Ia mouse alloantisera were produced in the following strain combinations by hyperimmunization of recipient mice with donor spleen and lymph node cells (21); anti-K^k, (A.TL × B10.A[5R]) F_1 anti-B10.A; anti-D^k, (C3H.SW × B10.A[2R]) F_1 anti-C3H; anti-Ia^k, A.TH anti-A.TL; anti-K^s, (A × A.AL) F_1 anti-A.TL (this serum was a kind gift from Dr. D. Shreffler, Washington University, St. Louis, Mo.); anti-D^s, A.TH anti-A.SW and anti-Ia^s, A.TL anti-A.TH. The anti-Ia^k and anti-Ia^s sera were rendered specific by in vivo absorption in B10.S($H\text{-}2^s$) and B10.BR($H\text{-}2^k$) mice, respectively (22). A rabbit antiserum against the X5563 IgG_{2a} myeloma protein (RAMP) was produced as previously described (23) and shown by immunodiffusion to react with all classes of mouse immunoglobulin. The congenic anti-Thy 1.2 sera, [$A^{Thy1.1}$ × AKR-$H\text{-}2^b$] F_1 anti-ASL1 (generously supplied by Dr. U. Hämmerling, Sloan-Kettering Institute, New York, N.Y.) and AKR/J anti-AKR/Cum were used for the identification and depletion of T cells, respectively.

Preparation of AEF. AEF was produced by a procedure similar to that previously reported (14) by using *H-2* congenic mouse strains. B10.BR($H\text{-}2^k$) lymphocytes were allogeneically activated against $H\text{-}2^s$ antigens by intravenous injection of irradiated (800 rad) B10.S($H\text{-}2^s$) recipients (2–3 mo old) with $1\text{–}2 \times 10^8$ B10.BR donor (1–2 mo old) thymocytes. 5 days later, approximately 90% of recipient spleen cells were found to be activated T cells of donor *H-2* haplotype, as tested with anti-Thy 1.2 and anti-K^k sera in the presence of rabbit complement by a dye exclusion cytotoxicity assay (21). Each recipient spleen yielded about $1.5\text{–}2.5 \times 10^7$ cells. Cell suspensions were prepared at 2×10^7 cells/ml in serum-free Click's medium (24) supplemented with 4 mM glutamine and 5 ×

10^{-5} M 2-mercaptoethanol immediately before use. Equal numbers (10^7) of activated responder B10.BR cells and normal irradiated (3,000 rad) stimulator B10.S spleen cells were cocultured in a vol of 1 ml by rocking in a moist 5% CO_2 atmosphere. Allogeneic supernates (AEF) of 20–24 h MLR were harvested by centrifugation at 2,500 rpm for 15 min, filtered through an 0.22-μm Millipore filter (Millipore Corp., Bedford, Mass.) previously washed with serum-free Click's medium and stored in samples at −70°C for as long as 3 wk without any significant loss of activity. Both the in vivo and in vitro activation steps described here were found to be necessary to generate AEF activity as assayed below.

A technique has been developed which allows a direct correlation to be made between the selective expression of Ia determinants amongst functional lymphocyte subpopulations with a particular functional role for Ia antigens in the regulation of the immune response by either T cells (20) or B cells (25). The method is dependent upon the elimination of specific functional lymphocyte subpopulations bearing surface Ia antigens by treatment with anti-Ia serum and complement (18, 20, 25). This negative selection procedure was adapted for the identification of the cellular origin of AEF.

Ia-negative activated responder B10.BR spleen cells were prepared by treatment of 10^8 cells with 1 ml of anti-Ia^k diluted 1:5 for 20 min at room temperature. Cells were centrifuged and resuspended in 3 ml of rabbit complement diluted 1:9 and incubated for a further 45 min at 37°C, then washed twice and resuspended in serum-free Click's medium before culture. Approximately 10–25% of the cells were specifically lysed by this procedure. T-cell-depleted normal stimulator B10.S spleen cells were prepared under similar conditions by reactivity with anti-Thy-1.2 (AKR/J anti-AKR/Cum) and rabbit complement. The latter treatment resulted in 25–35% specific cell lysis.

Immunoadsorbent Fractionation of AEF. IgG fractions were isolated from 2–3 ml of various mouse alloantisera by ammonium sulfate precipitation and DEAE cellulose chromatography in 0.04 M potassium phosphate buffer, pH 8.0. Approximately 6 mg of IgG was coupled to cyanogen-bromide activated Sepharose 4B (Pharmacia Fine Chemicals Inc., Piscataway, N.J.) (26) and the conjugate packed in a 1 × 3-cm column. Each amount of conjugated IgG was serologically estimated to be in excess of the amount of antigen potentially present in AEF. A similar column was formed by using about 10 mg of the IgG fraction from RAMP, prepared by passage through DEAE cellulose in 0.01 M phosphate buffer, pH 7.4, containing 0.15 M NaCl. Antigen-coated columns were also formed by coupling 10 mg of either DNP_{11}-KLH or KLH to CNBr-activated Sepharose 4B.

AEF was added to and incubated on a column during 1 h at 4°C, unbound components were then eluted with phosphate-buffered saline (0.02 M phosphate, pH 7.4, 0.15 M NaCl), filtered through a 0.22-μm Millipore filter (Millipore Corp.) and immediately added at the appropriate dilution to in vitro spleen cell cultures.

In Vitro Antibody Responses. Primary anti-SRBC and secondary anti-DNP PFC responses were evaluated in a microculture assay similar to that devised by Armerding and Katz (15). Spleen cells (10^6) derived from either nonimmunized or DNP-KLH-primed donors were suspended in Click's medium containing 5% fetal calf serum (Click-FCS) and cultured in a 0.3-ml vol in a Mictrotest II Falcon tissue culture plate (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) for either 5 days with 3 × 10^6 SRBC or 6 days with 1 ng/ml of DNP-KLH. AEF at concentrations between 10 and 50% was introduced into appropriate cultures before the addition of spleen cells. T-cell-depleted, DNP-KLH-primed spleen B cells were prepared by treatment of 10^8 spleen cells with 1 ml (diluted 1:5) of an anti-Thy 1.2 serum (AKR/J anti-AKR/Cum) for 20 min at room temperature and 3 ml of rabbit complement for a further 45 min at 37°C, then washed three times with Click-FCS and added to the cultures. This treatment resulted in 30–40% cell lysis. The surviving cell population was found to contain routinely less than 3% of Thy-1.2-bearing cells, as assayed by immunofluorescence by using a fluorescein-conjugated AKR/J anti-AKR/Cum serum and a Leitz Orthoplan fluorescence microscope (E. Leitz, Inc., Rockleigh, N.J.) equipped with Ploem illumination.

Cells from triplicate wells were pooled, harvested, and their PFC responses analyzed by the Cunningham and Szenberg modification of the Jerne assay (27). SRBC were used as indicator cells for the primary anti-SRBC PFC response. Anti-DNP indirect PFC were developed by using RAMP and 2,4,6-trinitrophenyl-coupled burro erythrocytes (20).

TABLE I

Strain Distribution Analysis of Helper Cell Function of AEF in an In Vitro Primary Anti-SRBC Response

Strain	H-2 Regions I									Spleen cells (1×10^6)	Antigen (3×10^6 SRBC)	Direct PFC/10^7-cultured cells	
	K	A	B	J	E	C	S	G	D			No AEF	AEF(50%)
B10.BR	k	k	k	k	k	k	k	k	k	Untreated	–	176 (1.22)	295 (1.18)
											+	3,143 (1.07)	9,386 (1.05)
										Anti-Thy 1.2 treated	–	221 (1.20)	370 (1.18)
											+	294 (1.22)	4,987 (1.07)
B10.S	s	s	s	s	s	s	s	s	s	Untreated	–	338 (1.13)	798 (1.06)
											+	2,742 (1.08)	6,938 (1.06)
										Anti-Thy 1.2 treated	–	80 (1.06)	199 (1.11)
											+	246 (1.24)	5,845 (1.03)
B10.A	k	k	k	k	k	d	d	d	d	Untreated	–	299 (1.07)	447 (1.12)
											+	3,590 (1.09)	9,878 (1.07)
										Anti-Thy 1.2 treated	–	124 (1.37)	286 (1.19)
											+	274 (1.24)	4,188 (1.08)
B10.A(4R)	k	k	b	b	b	b	b	b	b	Untreated	–	945 (1.11)	990 (1.16)
											+	7,965 (1.10)	16,356 (1.08)
										Anti-Thy 1.2 treated	–	98 (1.22)	545 (1.12)
											+	595 (1.14)	6,230 (1.08)
B10.D2	d	d	d	d	d	d	d	d	d	Untreated	–	358 (1.12)	597 (1.11)
											+	4,092 (1.06)	11,384 (1.05)
										Anti-Thy 1.2 treated	–	345 (1.19)	548 (1.12)
											+	442 (1.20)	5,897 (1.03)
B10	b	b	b	b	b	b	b	b	b	Untreated	–	267 (1.16)	346 (1.15)
											+	2,638 (1.10)	2,742 (1.08)
										Anti-Thy 1.2 treated	–	191 (1.36)	546 (1.14)
											+	294 (1.22)	647 (1.10)
B10.A(5R)	b	b	b	k	k	d	d	d	d	Untreated	–	297 (1.14)	297 (1.18)
											+	3,943 (1.07)	4,089 (1.08)
										Anti-Thy 1.2 treated	–	245 (1.22)	548 (1.12)
											+	294 (1.22)	647 (1.10)

Either 1×10^6 untreated or anti-Thy 1.2 (AKR/J anti-AKR/Cum) plus complement-treated cells were cultured in 0.3 ml of Click's medium (23) containing 5% FCS in the absence (–) or presence (+) of SRBC (3×10^6) and AEF (50%) for 5 days. Direct PFC were analyzed by the Cunningham and Szenberg method (27) and are expressed as geometric means with standard errors in parentheses.

Results

Strain Distribution Analysis of AEF Helper Cell Function in a Primary Response. The enhancement and helper T-cell replacing activity of AEF in an in vitro primary anti-SRBC response of spleen cells derived from several *H-2* haplotypes is presented in Table I. An insignificant indirect PFC response was obtained with these cells and is therefore not shown here. The genotypes of the *H-2* congenic inbred and recombinant strains tested are indicated. It is evident that AEF generated in a serum-free medium augments by approximately threefold the response of untreated spleen cells and reconstitutes the response of T-cell-depleted spleen B cells of both responder B10.BR origin and B10.S stimulator origin. These two properties have been previously reported (14) for an AEF prepared in a serum-containing medium and in an alternate strain combination, DBA/2 (H-2^d) activated against C3H(H-2^k), that allowed for stimulation across *H-2* as well as non-*H-2* differences. However, in this latter combination, AEF displayed preferential activity towards responder DBA/2 cells. It was only after

partial purification that this AEF worked efficiently with C3H stimulator cells.

The triggering of both the responder and stimulator PFC responses by AEF could be due to a nonspecific mitogenic signal, or AEF could be comprised of molecules of both B10.BR and B10.S origin. The former possibility is unlikely since AEF reconstituted the B10.A, B10.A(4R), and B10.D2 responses, but not the B10 nor B10.A(5R) responses. A similar result was observed for the response of C57B1/6 (14), which possesses the same *H-2* haplotype ($H\text{-}2^b$) as B10. It is possible that in the T-cell-depleted B10 and B10.A(5R) cultures, B cells may proliferate but may not differentiate into antibody-producing cells upon addition of AEF at the beginning of the culture period. Addition of AEF to similar cultures during the last 48–60 h of culture stimulate antibody production whereas these allogeneic supernates are not required to induce B-cell proliferation (28). However, use of either this procedure or addition of AEF both at the beginning and during the last 48 h of culture proved unsuccessful in attempts to elicit a B10 and B10.A(5R) response. These data suggest that the ability of AEF to provide T-cell helper function during interaction with unprimed B cells may be determined by genes localized in the *K* end of the *H-2* complex; no indication of whether *K* or *I* region genes control this effect can be inferred. The pattern of reactivity cannot be attributed to the distribution of known Ia antigenic specificities in these strains.

AEF Helper Cell Activity and Immunochemical Characterization in a Secondary Response. The helper cell replacing function of AEF was also assessed in a secondary in vitro T-cell-dependent system. Fig. 1 illustrates the activity of AEF in the secondary anti-DNP indirect PFC response of untreated and anti-Thy-1.2 (AKR/J anti-AKR/Cum) plus complement-treated spleen cells from B10.BR and B10.S DNP-KLH primed donors. The low direct PFC response (200 PFC/10^7 cultured cells) observed with untreated spleen cells is not shown. Untreated B10.BR DNP-KLH-primed spleen cells produced an indirect PFC response that was usually 500–1,000 PFC greater than that achieved by B10.S-primed cells. The response of each of these cell populations was not significantly increased by the addition of AEF at a final concentration of 50%, or at a concentration of 25% (results not shown). These concentrations of AEF each fully reconstituted the responses of anti-Thy-1.2-treated B10.BR and B10.S spleen cells. Approximately only half this level of reconstitution was achieved with AEF used at a 10% final concentration. However, AEF tested at the above concentrations did not restore the response of T-cell-depleted B10 cells (T. Delovitch, unpublished observations). Hence, this pattern of reconstitution of helper cell function in the secondary anti-DNP response paralleled that obtained for the primary SRBC response (Table I) and confirmed the lack of antigenic specificity of AEF.

Based on the ability of a cross-reactive anti-Ia serum to absorb AEF activity, and on the molecular size of the active AEF components, Armerding et al. (16) concluded that AEF contains Ia antigens. However, the serum used by these investigators also contained potential cross-reactive antibodies against *H-2K* antigens. No evidence was presented to determine whether the absorptive capacity of this serum was due to its anti-Ia or its anti-*H-2K* activity. For this reason, the role of Ia antigens in an AEF induced in vitro immune response was examined.

The nature of AEF was determined by testing its activity after fractionation

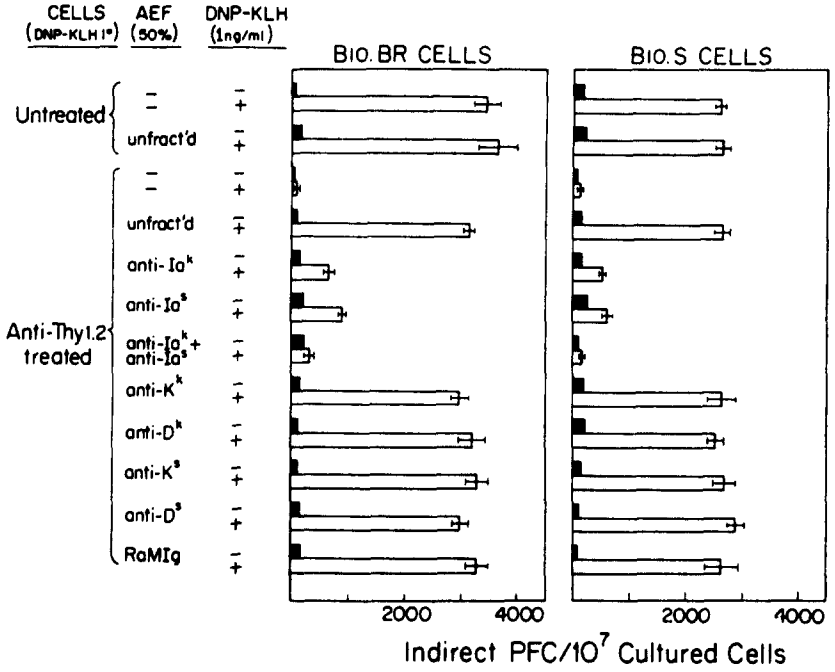

FIG. 1. Functional and immunochemical analysis of AEF activity in a secondary in vitro anti-DNP response. Culture conditions and anti-Thy 1.2 treatment were similar to that described in Table I. Untreated and anti-Thy 1.2-treated spleen cells from either B10.BR or B10.S DNP-KLH-primed mice were cultured with or without DNP-KLH (1 ng/ml) in the absence or presence of unfractionated or immunoadsorbent column fractionated AEF. Indirect PFC were evaluated after 6 days of culture by using trinitrophenyl-coupled burro RBC (19) and are presented as standard errors of the geometric mean. Sera used for immunoadsorption are as described in Materials and Methods.

on various immunoadsorbents. A concentration (50%) of AEF corresponding to the plateau of its activity was used after column fractionation. Similar data were obtained for both B10.BR and B10.S cell populations (Fig. 1). Absorption on an anti-Ia^k or on an anti-Ia^s column each resulted in a loss of 80% of AEF activity when compared to the activity of a similar concentration of unabsorbed AEF. The possibility that this absorption of AEF activity is due to a threshold effect is unlikely since the same results were observed with AEF at concentrations between 20 and 50% (results not shown). The anti-Ia sera used here, by virtue of their method of preparation, do not contain detectable anti-*H-2K* or anti-*H-2D* activity. Serial passage of AEF through these two anti-Ia immunoadsorbent columns completely abrogated its activity. By contrast, no AEF activity was removed by an anti-K^k, anti-D^k, anti-K^s, anti-D^s, or a polyvalent rabbit anti-mouse IgG (RAMP) column. These results confirmed those previously obtained for the primary SRBC response by using identical anti-Ia and anti-Ig columns (T. Delovitch, unpublished observations). By these criteria, it would appear that the active components of AEF consist, in part, of Ia antigens derived from both responder B10.BR and stimulator B10.S cells.

Cellular Origin of AEF. It has been generally assumed that AEF is a T-cell product because (*a*) AEF can replace T-cell-dependent helper function (14, 28);

(*b*) adherent cells are apparently not required for AEF production (D. Katz, personal communication) nor do they seem to produce it in significant amounts (28); (*c*) AEF has been postulated to be an inductive signal for the differentiation of antigen-sensitive B cells into antibody-forming cells (28); and (*d*) the allogeneic effect in vitro has been purported to be mediated by direct interaction of AEF with the responding B cells (14). However, in view of the above indications that Ia antigens derived from both responder and stimulator cells are molecular constituents of AEF, it is conceivable that AEF is not a T-cell product.

To test this possibility, the activity of AEF prepared from mixtures of subpopulations of responder and stimulator cells was analyzed. An assay was devised which selected for the generation of AEF activity by stimulator B cells in the MLR, and selected against the generation of AEF activity by responder T cells in the MLR. This experiment was considered feasible since responder T cells in the MLR are considered to be Ia negative as demonstrated by their capacity to respond after treatment with anti-Ia and complement, and because isolated B cells, which are Ia positive, stimulate strongly in an MLR (18, 29). The latter observations also tend to eliminate the responder T cell as a possible source of Ia antigens in AEF and raise the possibility that AEF Ia molecules may be derived from a B cell and/or macrophage of the stimulator cell population.

The negative selection procedure for the preparation of this AEF was performed as follows. B10.BR(H-2^k) thymocytes were activated in vivo against B10.S(H-2^s) cellular alloantigens as described (see Materials and Methods) and then reacted in vitro with anti-Ia^k and complement to deplete this responder population of Ia^k-positive cells. Normal B10.S stimulator spleen cells were treated with anti-Thy-1.2 and complement to eliminate any T cells which could potentially recognize residual Ia^k-positive cells in the responder spleen cell population. Both of these treatments were designed to minimize the production of responder Ia^k antigens and stimulator T-cell-derived Ia^s antigens in AEF. AEF was produced in 24 h mixed lymphocyte cultures of Ia-negative responder T cells and irradiated, T-cell-depleted, stimulator Ia^s-positive spleen cells. A strong MLR response is obtained with similar cultures (containing normal Ia negative responder T cells and irradiated T-cell-depleted Ia-positive spleen cells) maintained for 96 h (T. Delovitch, unpublished observations). The activity of this AEF was then tested for its ability to interact with either B10.BR or B10.S DNP-KLH-primed, T-cell-depleted, spleen B cells.

It is evident from Fig. 2 that AEF produced in the above manner (AEF-I) reconstituted the secondary anti-DNP response of B10.S T-cell-depleted-primed spleen cells but not B10.BR T-cell-depleted-primed spleen cells. By contrast, an AEF (AEF-II) derived from a MLR between irradiated, T-cell-depleted, stimulator B10.S spleen cells and activated responder B10.BR cells that were pretreated with anti-Ia^s and complement, which would not be expected to cause the lysis of these cells, reconstituted both the B10.BR and B10.S secondary anti-DNP responses. The latter result is similar to that obtained with AEF produced by the usual protocol by using untreated B10.BR responder cells and untreated B10.S stimulator cells (see Fig. 1). Thus, AEF-I, generated in the presence of Ia-negative responder T cells and T-cell-depleted stimulator cells (*a*) possesses a definite *H-2* haplotype restriction in its activity, and will be termed restricted AEF and (*b*) may be a B-cell product which can stimulate only B cells that bear

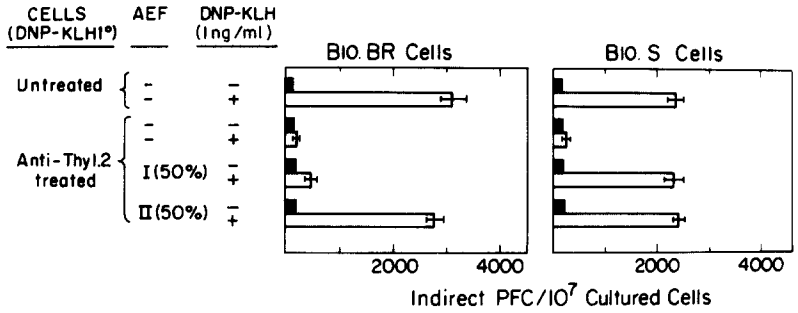

FIG. 2. Haplotype restriction of AEF activity. AEF activity was analyzed with supernates from mixtures of activated B10.BR responder cells, which were pretreated with either anti-Ia^k (AEF-I) or anti-Ia^s (AEF-II) plus complement, and anti-Thy 1.2 plus complement-treated irradiated B10.S stimulator cells. Culture conditions and evaluation of PFC were as noted in Fig. 1.

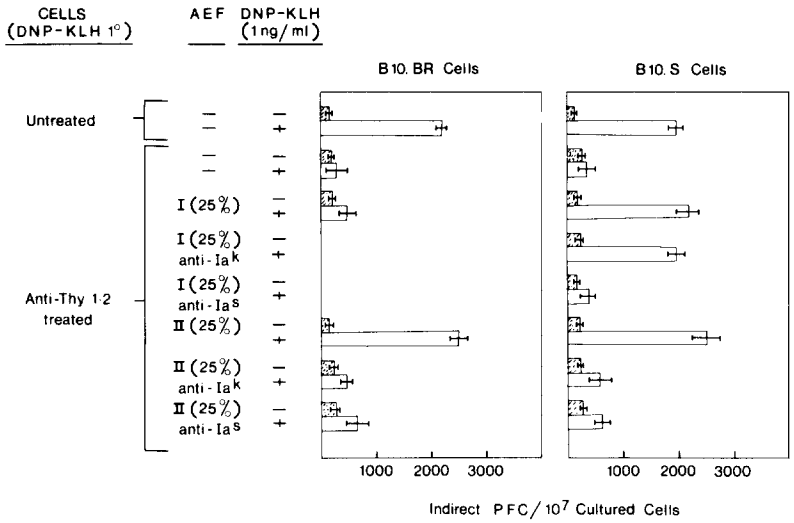

FIG. 3. Immunoadsorption of restricted AEF activity. AEF-I and AEF-II were produced as described in Fig. 2. Immunoadsorption on anti-Ia^k and anti-Ia^s columns, culture conditions, and evaluation of PFC were as described in Fig. 1.

similar Ia antigens. It should be noted, however, that macrophages present in the stimulator cell population may also potentially serve as a source of restricted AEF.

To examine further the possibility that restricted AEF Ia antigens are derived from a stimulator B cell, the removal of restricted AEF activity by anti-Ia^k and anti-Ia^s immunoadsorbent columns was tested. If the Ia antigen components of restricted AEF are derived from the B10.S ($H\text{-}2^s$) stimulator B cell (and/or macrophage), then the prediction would be that an anti-Ia^s column but not an anti-Ia^k column should adsorb its activity. As is demonstrated in Fig. 3, approximately 90% of the capacity of restricted AEF (AEF-I) to stimulate the secondary anti-DNP response of B10.S DNP-KLH-primed T-cell-depleted spleen B cells was eliminated by adsorption on an anti-Ia^s column but not adsorption on an anti-Ia^k column. However, both the anti-Ia^k and anti-Ia^s columns removed the

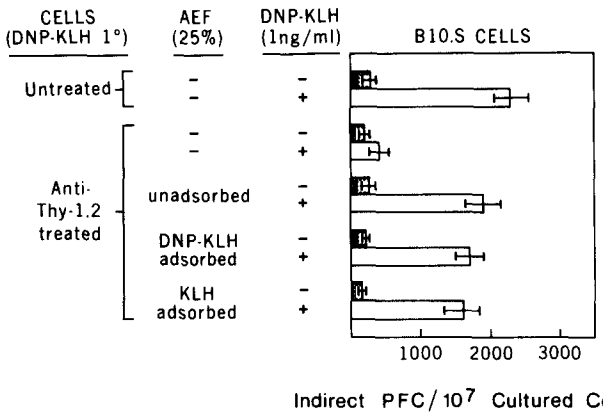

FIG. 4. Failure of antigen-immunoadsorbents to remove restricted AEF activity. The activity of restricted AEF was tested either before or after adsorption on a DNP-KLH or KLH-coated column.

activity of AEF-II produced by an MLR between anti-Ia^s and complement-treated activated B10.BR responder cells and T-cell-depleted, irradiated, B10.S stimulator spleen cells. This result is comparable to that obtained for the conventionally produced AEF (see Fig. 1).

The above data clearly indicate that whereas Ia antigens in AEF produced in the usual manner are products of both the responder cell and the stimulator cell populations, restricted AEF Ia molecules are derived only from the stimulator cells. The source of Ia antigens in the responder cell population is not known. Since the responder T cell in an MLR has been purported to be Ia negative, AEF Ia antigens derived from the responder cell population must be products of non-MLR responsive T cells and/or the low proportion (approximately 5%) of B cells in this population.

Role of Antigen in Restricted AEF. The activity of antigen-specific, T-cell-derived (12, 13) and macrophage-derived (30) Ia-positive factors may be removed by antigen immunoadsorbents. It is not clear whether the antigen-binding properties of these factors indicate that they are molecularly associated with antigen or otherwise serve as specific antigen receptors. To determine whether restricted AEF can bind antigen, the adsorption of its activity by either a DNP_{11}-KLH or KLH column was tested.

Passage of restricted AEF over each of these antigen columns failed to remove any of its helper activity in a secondary anti-DNP response of DNP-primed B10.S B cells (see Fig. 4). This result suggests that antigen is not a component of restricted AEF. However, it does not rule out the possibility that restricted AEF may become complexed with antigen in culture during the course of the 6-day in vitro secondary antibody response. Thus, it still may be possible that the B cell is triggered by antigen in association with Ia determinants of AEF in a manner similar to that previously described for a factor which mediates macrophage–T-cell interaction (30).

Discussion

To further delineate the central role that Ia antigens play in the regulation of T cell–B cell–macrophage collaboration during an immune response, an alloge-

neic effect factor was characterized with respect to its helper cell replacing activity, immunochemical nature, *H-2* haplotype specificity, genetic origin, and cellular origin.

Unlike an AEF previously generated by an *H-2* and non-*H-2* incompatibility (14), the AEF produced here across only an *H-2* difference is not genetically restricted in its activity as it enhances a primary antibody response of both responder and stimulator spleen cells. This AEF also provides helper cell function in a primary and secondary antibody response for both responder and stimulator T-cell-depleted spleen B cells. The pattern of reactivity of AEF in a primary response with B cells of several inbred and recombinant strains suggests that genes in the *K*, *I-A*, and *I-B* regions may control this type of interaction. These data differ from those reported for a soluble factor, produced by a similar procedure of alloantigen-activation across an *H-2* difference, that suppresses a mixed lymphocyte reaction (31). This suppressor factor expresses antigen specificity towards the responder cell and not the stimulator cell, and this phenomenon is apparently determined by the *I-C* subregion.

An immunochemical analysis of AEF activity established that Ia antigens, but neither *H-2* antigens nor immunoglobulins, represent an active component of AEF. These data, taken together with those suggesting the *K*, *I-A*, and *I-B* region control of AEF activity, imply that only one or both of the latter two regions may control this activity. Since an antigen-specific T-cell-derived suppressor cell factor seems to be comprised of Ia antigens determined by genes in the *I-J* subregion situated between the *I-B* and *I-E* subregions (32), it would appear that Ia determinants of helper and suppressor cell factors are controlled by different *I*-region genes. This idea derives additional support from the finding that an anti-Ia serum which inactivates allotype suppressor T cells does not affect helper T-cell activity (10). Nevertheless, no Ia moleculè with known specificities may as yet be assigned as a component of either a helper or suppressor cell factor.

Immunoadsorption on anti-Ia columns of the activity of AEF produced in the usual manner provided two interesting observations. First, AEF is comprised of Ia antigens derived not only from activated responder cells but also from the irradiated stimulator cells. Second, since about 75% of AEF activity was removed by each of the anti-Ia sera reactive with the responder and stimulator haplotypes and since AEF activity was completely removed by a combination of these two antisera, this implies that a complex is formed between AEF Ia antigens derived from the two haplotypes. If a complex between these antigens does exist, it is not clear whether (*a*) it represents a covalent or noncovalent interaction, (*b*) it plays a role, as such, in vivo during allogeneic lymphocyte interaction or, (*c*) it merely arises artifactually during an in vitro MLR from the formation of membrane vesicles released from the responder and stimulator cell populations. Further experimentation should resolve these possibilities.

The characterization of an AEF produced in an MLR between Ia-negative responder cells and T-cell-depleted stimulator cells confirmed the above data that AEF Ia antigens may also originate from the stimulator haplotype. More significantly, the stimulator B cell was implicated as one source of Ia antigens present in AEF. This AEF stimulated the secondary antibody response of T-cell-depleted B cells of the stimulator but not responder haplotype. While the genetic

origin of this haplotype restricted AEF and AEF generated by the usual procedure has not yet been mapped precisely within the *I*-region, it is clear that genetic differences exist in the patterns of helper cell replacing function displayed by these two AEF preparations. These reactivities may be explained by the different origins of the two factors. Restricted AEF is a product of the stimulator B cell and/or macrophage and may be able to help only B cells of its own haplotype, or of haplotypes sharing identical Ia antigens. By analogy, AEF produced in the usual manner contains Ia antigens derived from both the responder and stimulator cells, and may therefore be able to stimulate B cells of a wider range of *H-2* haplotypes which possess similar or cross-reacting Ia determinants.

Whether the stimulator B cell is a source of restricted AEF has not been difinitively determined. It is possible that this AEF is also derived from the residual stimulator T cells which constitute less than 3% of the T-cell-depleted stimulator cell population. Macrophages, which bear Ia determinants (33, 34) and produce a soluble Ia-positive helper factor that mediates T-cell macrophage interaction (30), may also be present in the stimulator cell population and serve as a potential source of restricted AEF. However, it has been reported that macrophages are apparently not required for AEF production, nor do they seem to produce it in detectable amounts (28). It is suggested, therefore, that the major source of restricted AEF is the stimulator B cell because of the high proportion of B cells in the stimulator cell population and because of the predominant expression of Ia antigens by B cells. While AEF produced in the usual manner also originates from the responder cell population, the source of Ia antigens in this population remains unknown. What is apparent is that the responder T cell does not produce the Ia antigens present in AEF.

Considerable evidence has indicated that allogeneic T cell–B-cell interaction during an immune response may occur via the specific recognition of Ia antigens on hapten-primed B cells by allogeneic T cells. For example, the allogeneic effect (35) is manifested by the recognition by allogeneic T cells of alloantigens (possibly Ia molecules) on hapten-primed B cells, which then potentiates an IgG secondary anti-hapten response to hapten on a heterologous carrier in the absence of syngeneic carrier-primed T cells. Similarly, (T,G)-A–L primed nonresponder spleen B cells can switch from IgM to IgG antibody production upon stimulation by allogeneic T cells but not syngeneic T cells (36). Ia antigens are considered to be the major determinants recognized in an MLR and graft-versus-host reaction (reviewed in references 1 and 18) and may also serve a recognitive role in the allogeneic effect. These findings are consonant with the idea that IgG antibody synthesis by hapten-primed B cells may be elicited in an antigen-nonspecific fashion via the recognition of their Ia antigens by allogeneic T cells. By contrast, it appears that a syngeneic T cell-B cell interaction, which may also be mediated by the recognition of Ia antigens, is requisite to promote the switch in an antigen-specific response from IgM to IgG antibody production (25).

The mechanism by which the recognition of allogeneic Ia antigens occurs in an IgG secondary response and its relationship to the process of normal B-cell activation is still not well defined. From the data presented here, it is apparent that AEF is an Ia-positive product of a B cell and/or macrophage which can activate B cells directly for IgG antibody synthesis in the absence of syngeneic

carrier-primed T cells. This type of activation may occur as a result of a structural modification of Ia antigens in AEF after their release from the surface of a B cell or macrophage. One type of modification may occur via the association of a T-cell-derived Ia-negative component with an Ia molecule in AEF. It is possible that the B cell possesses an acceptor molecule for this postulated T-cell product. Thus, a primed B cell may need to recognize both syngeneic Ia antigens and a T-cell Ia-negative signal to be suitably triggered in the presence of antigen to IgG antibody synthesis. If this is the case, it is possible that similarly modified antigen-specific Ia-positive B cell or macrophage-derived signals may mediate the process of normal B-cell activation. A further study of the mechanism and target of action of AEF, and its role in B-cell activation, is currently in progress.

Summary

A soluble allogeneic effect factor (AEF) was produced by using *H-2* congenic mouse strains and a serum-free cell culture medium. An AEF derived from untreated activated responder cells and irradiated stimulator cells provided helper cell function in a primary and secondary antibody response for both T-cell-depleted responder B cells and stimulator B cells. This interaction may be determined by genes situated in the *I-A* and *I-B* regions: additional *K*-region control was not excluded. Ia antigens, but neither *H-2* nor Ig determinants are molecular constituents of AEF. The active components of this AEF consist, in part, of Ia antigens derived from both the activated responder cell population and irradiated stimulator cell population.

An AEF derived from Ia negative responder cells and irradiated T-cell-depleted stimulator cells helps a secondary antibody response of T-cell-depleted stimulator B cells but not responder B cells. This genetically restricted AEF contains Ia antigens determined by the stimulator haplotype but not the responder haplotype. The priming antigen, DNP-keyhole limpet hemocyanin, is not a component of restricted AEF. The data suggest that restricted AEF may be a product of a stimulator B cell and/or macrophage. They support the hypothesis that the recognition by allogeneic T cells of Ia antigens on B cells activates the B cell to IgG antibody production.

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