CONSTRUCTION OF T CELL HYBRIDOMAS SECRETING ALLOGENEIC EFFECT FACTOR*

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Allogeneic effect factor (AEF)[1] is a soluble mediator secreted into supernates of short-term secondary mixed lymphocyte cultures (MLC) of in vivo alloantigen-primed T cells (1–7). Although studied and characterized initially in terms of its ability to activate B cells to develop antibody responses in vitro to particulate and soluble antigens in the absence of helper T cells (1–7), recent studies in our laboratory have focused on some very interesting T cell-activating properties of AEF (8–13). These latter studies have shown that AEF (*a*) is highly mitogenic for unprimed T cells, (*b*) can trigger the differentiation of unprimed T cells, in the absence of exogenous antigen, into cytotoxic T lymphocytes (CTL), which display the Lyt-2^+ phenotype, preferentially lyse *H-2*-identical target cells, and are directed against antigen(s) determined by the *K* region of the murine major histocompatibility complex, and (*c*) stimulates the differentiation of unprimed T cells into responding cells of the Lyt-1^+ phenotype that can be restimulated in secondary syngeneic mixed lymphocyte reactions (MLR) that are directed against antigen(s) determined by the *I* region of the *H-2* complex (8–13). These latter properties of AEF, namely its ability to trigger differentiation of unprimed T cells into functional effector cells, distinguishes this biologically active mediator from those lymphokines, such as T cell growth factor, that are now designated interleukin-2 (IL-2); IL-2 moieties are characterized biologically by their ability to support long-term growth of primed, fully mature T lymphocytes previously activated by antigen or otherwise (14).

This heterogeneity of biological activities displayed by AEF-containing MLC supernates raises the question of whether the same or different molecules mediate these various effects. Recent biochemical analysis has shown that the AEF-mediated B cell- and T cell-activating properties cochromatograph on sizing gels and coelectrophorese when subjected to isoelectric focusing (D. H. Katz and A. Altman. Manuscript in preparation.). Although suggestive, such biochemical criteria are still inadequate

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[1] *Abbreviations used in this paper:* AEF, allogeneic effect factor; C, complement; CTL, cytotoxic T lymphocytes; DMEM, Dulbecco's minimum essential medium; EHAA, Eagle-Hank's amino acid medium; FcR, Fc receptors; FCS, fetal calf serum, [^{3}H]TdR, tritiated thymidine; IBF, immunoglobulin-binding factor; IL-2, interleukin-2; MLC, mixed lymphocyte culture; MLR, mixed lymphocyte reaction; PFC, plaque-forming cells; PNA^-, peanut agglutinin-nonagglutinable; SRBC, sheep erythrocytes; TNP, trinitrophenyl.

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to allow conclusions concerning molecular heterogeneity or homogeneity with regard to the distinct biological activities of AEF. In order to address this and other questions more directly, and to provide a more uniform source of AEF molecules for further purification and analysis, we have utilized the technique of somatic cell hybridization to construct T cell hybridomas secreting AEF. This report describes the characteristics of such hybridomas and the AEF secreted by them.

Materials and Methods

Mice. All inbred mice employed in these studies were obtained from the Scripps Clinic and Research Foundation (SCRF) mouse breeding colony, The Jackson Laboratory (Bar Harbor, Maine), or from Simonsen Laboratories (Gilroy, Calif.).

Preparation of AEF. AEF was prepared in the conventional manner as described previously (1, 9). 1×10^8 thymocytes from young (4- to 6-wk-old) DBA/2 donors were mixed with an equal number of irradiated (1,500 rad) spleen cells from (C3H × DBA/2) F_1 hybrid mice ($C3D2F_1$) and injected intravenously into irradiated (650 rad) DBA/2 recipient mice. The alloantigen-activated T cells recovered from the spleens of these mice 7 d later were co-cultured with fresh irradiated $C3D2F_1$ stimulator spleen cells. Responder and stimulator cells were pretreated with 10 μg/ml phenylmethylsulphonyl fluoride to inhibit the activity of serine proteases that tend to degrade AEF (6) and cultured for 18–24 h at 1×10^7/ml each in serum-free RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 1×10^{-4} M 2-mercaptoethanol. Cultures were maintained in a Mishell-Dutton gas mixture on a rocking platform. The culture supernate was obtained by centrifugation (2,500 rpm, 15 min), filter sterilized, and stored at −70°C until used.

Construction of AEF-secreting Hybridomas. T cell blasts activated to alloantigen in vivo and secondarily stimulated in vitro as described above were recovered after 18 h in culture and separated in Ficoll-Hypaque in Dulbecco's minimum essential medium (DMEM): phosphate-buffered saline solution prepared as described elsewhere (15). Cells recovered from the interface after centrifugation were predominantly blastlike in nature and were used for hybridization.

The parental cell line used for hybridization was the AKR-derived T cell line, BW 5147 obtained from Dr. Robert Hyman of The Salk Institute, La Jolla, Calif. This line is hypoxanthine guanine phosphoribosyl transferase negative, and displays the following cell surface phenotype (16): $H\text{-}2^{k+}$, Thy-1.1^+, Ig^-, Ia^-, Lyt-1^+, Lyt-2^-.

Cell fusion was performed according to Gefter et al. (17) with some modifications. Parental tumor cells were washed twice in DMEM, and 2×10^6 tumor cells were mixed with 1×10^7 activated T cell blasts. The cell suspension was centrifuged, and all medium was carefully removed by aspiration. The cell pellet was then resuspended in 0.2 ml of 30% polyethylene glycol 1,000 in DMEM. Cells remained in this solution for a total of 8 min, during which time they were centrifuged for 6 min at 1,000 rpm (ambient temperature). At the end of 8 min, 5 ml of DMEM was added to the tube, and the cells were washed twice in this medium. They were resuspended in DMEM supplemented with 10% fetal calf serum (FCS) and incubated overnight at 37°C in a 10% CO_2 in air atmosphere. The next day, the cells were pelleted by centrifugation and resuspended in 30 ml of FCS-supplemented DMEM containing hypoxanthine (1×10^{-4} M), aminopterin (4×10^{-6} M), and thymidine (1.6×10^{-5} M), and were distributed into individual wells of 96-well flat-bottom microplates. Cultures were fed after 1 wk by the addition of one drop of this same medium without aminopterin.

Screening of hybridoma supernates was carried out by incorporating multiple dilutions of each given supernate tested into primary in vitro antibody responses to sheep erythrocytes (SRBC) or trinitrophenyl (TNP)-derivatized SRBC (see below).

Biological Assays for AEF Activity

IN VITRO ANTIBODY RESPONSE SYSTEM. AEF preparations and hybridoma supernates were tested for their ability to reconstitute primary in vitro antibody responses against SRBC or TNP-SRBC of anti-Thy 1.2 serum plus complement (C)-treated DBA/2 or A/J spleen cells. The methods for establishing such cultures and for analyzing the resulting SRBC- or TNP-specific plaque-forming cells (PFC) were identical with those described previously (1).

CTL CULTURE SYSTEM. Spleen cell suspensions of normal, unprimed mice were prepared and depleted of erythrocytes by NH_4CL treatment as described previously (9). The cells were resuspended in Eagle-Hank's amino acid (EHAA) medium (18) supplemented with 5×10^{-5} M 2-ME and 5% heat-inactivated FCS and cultured in Cluster[24] tissue culture plates (Microbiological Associates, Walkersville, Md.) at 7.5×10^6 cells per well in 2 ml of medium containing various concentrations of AEF or hybridoma supernate as described in Results. The cultures were maintained for 5 d in a 5% CO_2 incubator and then assayed for their cytolytic activity. The cytolytic activities in such cultures were determined using the Thy-56 lymphoma (BALB/c, *H-2*^{d}) labeled with ^{51}Cr as described in detail elsewhere (19).

PROLIFERATION ASSAY. $2.5–5 \times 10^5$ spleen cells were cultured in triplicate in flat-bottom wells of Microtest II tissue culture plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) in 0.2 ml of complete EHAA medium containing various concentrations of AEF or hybridoma supernate. Cultures were maintained for 5 d, pulsed for the final 16 h with 1 μCi tritiated thymidine [^{3}H]TdR, New England Nuclear, Boston, Mass.), and harvested on a cell harvester (Skatron A.S. Lierbyen, Norway) (20).

Antisera and Cytolytic Depletion. Anti-Thy-1.1 and Thy-1.2 antibodies were prepared as described in detail previously (21). A.TL anti A.TH (anti-Ia^s) and B10.A anti-B10 (anti-*I-A*^{d}) were prepared as previously described (13). A similar immunization regimen was used to prepare B10.BR anti-B10.D2 (anti-*H-2*^{d}) and B10 anti-B10.BR (anti-*H-2*^{k}) antibodies. All of these reagents had titers of 1:1,000 or higher in a C-dependent microcytotoxicity assay (22).

Analysis of cell surface phenotypes of hybridomas was carried out in a C-dependent microcytotoxicity assay previously described from this laboratory (22).

Results

AEF-secreting T Cell Hybridomas Replace Helper T Cells in Primary In Vitro Anti-SRBC Responses of DBA/2 B Cell Suspensions. After fusion, hybrids were detected in 115 of 288 wells plated, and the supernates from these positive wells were screened for B cell-activating capacities. Approximately one-half of these supernates manifested little or no positive biological effects, about one-third displayed low-to-moderate biological activity, and a small number demonstrated rather potent biological effects when tested, as shown below.

The experiment portrayed in Fig. 1 illustrates the activities of two high AEF-secreting hybridomas in terms of their B cell-activating properties. In the left panel are shown the good in vitro responses of untreated DBA/2 spleen cells to SRBC and the abrogation of such responses after treatment of such cells with anti-Thy-1.2 plus C. In the right panel, the vertical bar illustrates the capacity of a conventional AEF preparation to restore totally the responses of T cell-depleted B cells after stimulation with SRBC. In addition, the B cell-activating effects of five different hybridoma supernates, tested at three different concentrations each, are illustrated. The hybridoma supernates tested were obtained in serum-free conditions by washing the cultured hybridoma cells, replating them in serum-free medium, and harvesting the culture supernates after 24 h. It is clear that supernates from those hybridomas designated 27 and 34 display strong B cell-activating effects, which dilute out in a dose-related fashion. Supernate from the hybridoma designated hybridoma 18 displayed modest B cell-activating effects only at the highest concentration tested, whereas supernates from hybridomas 31 and 10 are negative for such activity. Although not shown, culture supernates from the unfused parental line, BW 5147, likewise failed to display any B cell-activating properties in these cultures. Hybridomas 27 and 34 were selected for further experimentation and have now been maintained in culture for ~1 yr, retaining their biological activity.

Surface Phenotype Analysis of AEF-secreting Hybridomas. Hybridomas 27 and 34 were

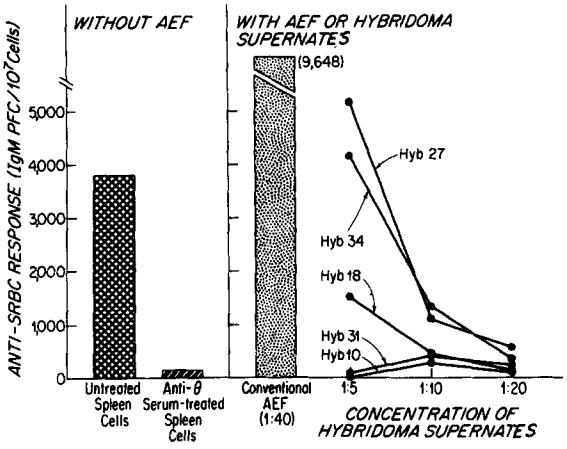

FIG. 1. AEF-secreting T cell hybridomas (Hyb) replace helper T cells in primary in vitro anti-SRBC responses of DBA/2 cell suspensions. DBA/2 spleen cells, either untreated (1.0×10^6 cells/well) or depleted of T cells by previous treatment with anti-θ serum plus C (0.5×10^6 cells/well) were cultured for 5 d in the presence of stimulating SRBC either without AEF added to the culture (left panel) or together with either conventional AEF or potential AEF-containing hybridoma supernates at the concentrations indicated (right panel). The data are presented as SRBC-specific IgM PFC/10^7 cells determined from triplicate cultures of each type. Control cultures consisting of either untreated or T cell-depleted spleen cells cultured in the absence of any stimulating SRBC yielded PFC levels ranging from 0 to 45 irrespective of whether AEF or AEF-containing hybridoma supernates were present in such cultures.

analyzed for expression of Thy-1 and *H-2* markers in a C-dependent microcytotoxicity assay. The results of such analyses demonstrated clearly that these are both somatic cell hybrids as evidenced by their expression of antigens encoded by both Thy-1 alleles and the *H-2* antigens characteristic of each of the respective partner cells fused.

Biological Effects on B and T Lymphocytes of AEF Hybridoma Supernate Obtained After Alloantigen Restimulation. Because the AEF hybridomas were derived from T cell blasts generated after stimulation by alloantigens, we were interested in determining whether the hybridomas themselves displayed any responsiveness to additional stimulation with the original inducing alloantigen. Thus, cells of hybridoma 34 were cultured overnight in the presence of equal numbers of irradiated C3D2F$_1$ target spleen cells, and the supernate was recovered from such stimulated hybridoma cultures and analyzed for biological activities on both B and T lymphocytes.

As shown in Fig. 2, the supernate from such restimulated hybridoma cells displayed excellent B cell-reconstituting effects in in vitro antibody responses to TNP-SRBC (left panel), and perfectly clear T cell-activating properties as manifested by its ability to stimulate development of CTL in the absence of exogenous antigen (middle panel), and direct proliferation of unprimed T lymphocytes (right panel). It should be noted that these activities were obtained with a concentration of hybridoma supernate (1:20) that is well below the activity threshold of supernates from the same hybridoma not restimulated with alloantigen (see also Fig. 1). Furthermore, it should be noted that the culture supernate of the unstimulated hybridoma failed to display B cell- or T cell-activating properties in this experiment when tested at the same dilution.

To ascertain whether the enhanced biological activity displayed by the culture supernate of the C3D2F$_1$-stimulated hybridoma reflects an antigen-specific event, we

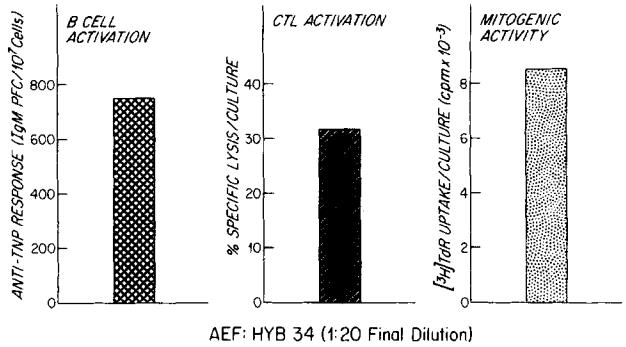

FIG. 2. Biological effects on B and T lymphocytes of AEF hybridoma (Hyb) supernate obtained after alloantigen restimulation. AEF:Hyb 34 was cultured for 24 h in the presence of an equal number of fresh, irradiated (1,500 rad) C3D2F$_1$ stimulator cells. The supernate recovered from such stimulated hybridoma cultures was tested at a final dilution of 1:20 for its B cell- and T cell-activating properties. B cell-activating properties were measured in terms of capacity to stimulate T cell-depleted DBA/2 spleen cells to respond in vitro to TNP-SRBC. The data are shown in the far left panel as TNP-specific IgM PFC/10^7 cells; control cultures of the same cells stimulated with TNP-SRBC in the absence of Hyb 34 supernate yielded no detectable TNP-specific PFC. The T cell-activating properties of AEF:Hyb 34 supernate at this dilution were measured by stimulation of self-specific CTL (middle panel) and direct mitogenic activity on unprimed T lymphocytes (right panel), using assay systems as described in Materials and Methods. The mitogenic activity is expressed as net counts per minute per culture in experimental cultures after subtraction of background counts per minute in unstimulated cultures. Background levels for CTL and mitogenic activities, were 6.9% lysis and 5,000 cpm, respectively.

Supernates from AEF:Hyb 34 cells not subjected to alloantigen restimulation failed to display significant biological activities at a final dilution of 1:20.

performed the following experiment: cells of hybridomas 27 and 34 were cultured for 24 h in the absence or presence of 4×10^5 irradiated spleen cells of either the specific ($H\text{-}2^k$) or an unrelated ($H\text{-}2^b$) target haplotype and their level of [^{3}H]TdR uptake was measured after a 4-h terminal pulse. Despite the high level of spontaneous [^{3}H]-TdR uptake by the unstimulated hybridoma cells, a specific alloantigen-dependent stimulation could readily be observed. Thus, the net stimulation of hybridomas 27 and 34 was 21,971 and 17,897 cpm, respectively, by specific CBA ($H\text{-}2^k$) stimulator cells and only 5,132 and 3,351 cpm, respectively, by the unrelated C57BL/6 ($H\text{-}2^b$) stimulator cells. This reflects a 4.2- to 5.3-fold difference in net stimulation by the specific vs. the unrelated spleen cell targets.

Inhibition of B Cell-activating Properties of AEF Hybridoma Supernate by Anti-Ia Antibodies. One of the prominent distinguishing features of AEF, as compared with many other nonspecific lymphokines, is the fact that determinants encoded by genes of the *I* region of the *H-2* complex are present on the biologically active AEF molecules (2). In order to verify that the AEF molecules detected in the T cell hybridoma supernates presented in this study were likewise Ia-positive, we investigated whether the activity of such molecules would be sensitive to the effects of anti-Ia antibodies.

The experiment summarized in Fig. 3 demonstrates that this is, indeed, the case. Anti-Thy-1.2-serum-treated spleen cells of A/J mice were used as responder cells in this experiment because they are totally disparate at the *I* region from the DBA/2 strain of origin of the AEF-secreting hybridomas. Moreover, A/J mice are *H-2* identical with the B10.A mice used for preparation of the anti-$I\text{-}A^d$ serum employed,

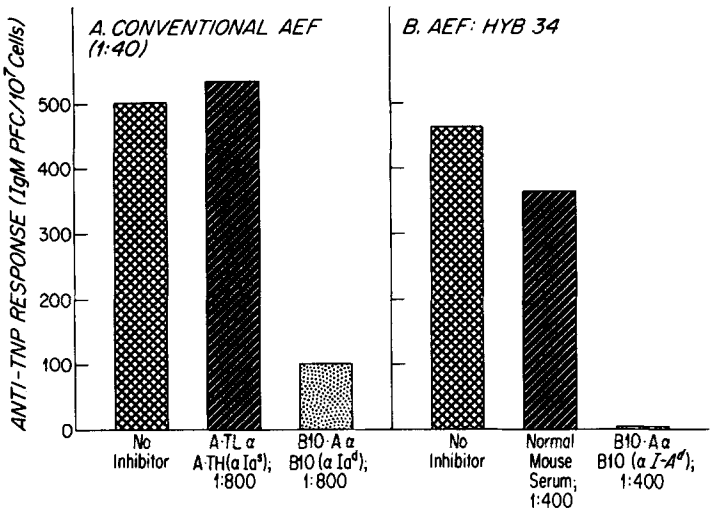

FIG. 3. Inhibition of B cell-activating properties of AEF hybridoma (Hyb) supernate by anti-$I\text{-}A^d$ antibodies. Unprimed A/J spleen cells depleted of T lymphocytes by treatment with anti-θ serum plus C were cultured (0.5×10^6 cells/well) for 5 d in the presence of TNP-SRBC as stimulating antigen and either conventional AEF (final concentration 1:40, panel A) or supernate from AEF: Hyb 34 (final concentration 1:5, panel B). Cultures contained either no inhibitor serum, normal mouse serum, anti-Ia^s, or anti-$I\text{-}A^d$ serum at the final concentrations indicated. The data are presented as TNP-specific IgM PFC/10^7 cells assayed in triplicate cultures of each type. Control cultures of T cell-depleted A/J spleen cells cultured in the presence of TNP-SRBC but without any source of AEF yielded no detectable TNP-specific PFC response. Other control cultures of untreated A/J spleen cells produced 516 TNP-specific PFC in response to TNP-SRBC, and this response was not inhibited in the presence of anti-$I\text{-}A^d$ serum.

and this serum was devoid of any reactivity with A/J spleen cells in a C-dependent microcytotoxicity assay (not shown).

As shown in panel A, cultures of such T cell-depleted A/J spleen cells exposed to a conventional AEF preparation develop good primary TNP-specific in vitro responses to TNP-SRBC. Incorporation of anti-Ia^s antibodies has no effect on the capacity of this AEF preparation (also derived from DBA/2-activated T cells) in stimulating such B cell responses. In contrast, incorporation of antibodies specific for $I\text{-}A^d$ determinants substantially inhibits the activity of this AEF preparation on the A/J responder B cells.

As shown in panel B, AEF activity of serum-free supernate of hybridoma 34 is not significantly affected by the incorporation of normal mouse serum of B10.A origin, whereas incorporation of anti-$I\text{-}A^d$ antibodies at the same concentration totally abolishes the B cell-activating properties of this AEF-secreting hybridoma. Control cultures of untreated (i.e., T cell-containing) A/J spleen cells developed good responses to TNP-SRBC, and these responses were not inhibited by the same anti-$I\text{-}A^d$ antibodies (Fig. 3, legend).

Cloning of Hybridoma 34. Hybridoma 34 was cloned by plating an average of 0.8 hybridoma cells per flat-bottom microtiter well (200 μl) in the presence of heavily irradiated BALB/c thymocytes, which served as feeder cells. Growth of hybridoma cells was detected in 75% of the wells after 10–14 d, and, of these, about two-thirds contained single cell clones. These clones were transferred to 16-mm wells, grown to

confluence, and their supernates were tested for induction of self-reactive CTL at a 1:10 final dilution. About 30% of the supernates displayed biological activity as evidenced by the resultant CTL activity, which was significantly above the background level of ^{51}Cr release. Furthermore, the culture supernates derived from some of these clones were mitogenic for peanut agglutinin-nonagglutinable (PNA^-) thymocytes, indicating that the hybridoma-derived AEF is indeed mitogenic for unprimed T cells. Thus, whereas the level of [^{3}H]TdR uptake by 2×10^{-5} unstimulated PNA^- thymocytes in a 5-d assay was 171 cpm, stimulation by the supernate of one of the clones was 3,247 and 14,716 cpm at 1:20 and 1:10 dilutions, respectively.

Cell Sorter Analysis of Ia Determinants on AEF-secreting Hybridomas. In view of our findings that conventionally derived AEF bears Ia determinants (2) and that the biological activity of both the conventional and the hybridoma-derived AEF preparations is inhibited by specific anti-Ia antibodies, it was of importance to determine whether the hybridoma cells express Ia antigens of the normal T cell partner, i.e., Ia^d. Thus, parental and cloned hybridoma cells were incubated with B10.A anti-B10 (anti-Ia^d) or A.TL anti-A.TH (anti-Ia^s) sera, stained with a fluorescent rabbit anti-mouse Ig serum, and subjected to analysis on a cell sorter (FACS II; B D FACS Systems, Mountain View, Calif.) (Table I). The intensity of fluorescence by the anti-Ia^d-treated cells was significantly higher than that by the anti-Ia^s-treated ones for the parental hybridoma 34 cells and for two of its clones (34.1 and 34.2), whereas another clone (34.3) did not display a specific fluorescence pattern. These results indicate that at least some of the hybridoma cells express Ia determinants.

Discussion

The technique of somatic cell hybridization as refined by Köhler and Milstein for immunologically relevant cells (23), has provided a revolutionary tool for studies on lymphocyte biology. This technology, developed initially for the construction of hybrid cell lines of the B cell lineage, subsequently was extended to the construction of T cell hybridomas by fusion of either normal or activated T cells with established T lymphoma lines (16, 24, 25). Although the success of hybridization with such T

TABLE I

FACS-II Analysis of Ia Determinants on an AEF-secreting Hybridoma and its Cloned Cell Lines

Cells	Cells in fluorescence channels 200–1,000		
	Anti-Ia^s	Anti-Ia^d	Δ
	% of total cells		
Hybridoma 34	49.9	70.7	20.8
Clone 34.1	14.4	44.1	29.7
Clone 34.2	46.4	73.3	26.9
Clone 34.3	56.9	59.3	2.4

Cells (5×10^6/ml) were incubated in the cold with 200 μl of a 1:10 dilution of either B10.A anti-B10 (anti-Ia^d) or A.TL anti-A.TH (anti-Ia^s) sera followed by 200 μl of a 1:100 dilution of a fluorescein-conjugated rabbit anti-mouse immunoglobulin (30 min each), washed three times, and analyzed on the cell sorter (FACS II; Becton, Dickinson & Co). The FACS II was calibrated using 5-μm latex microspheres (light scatter peak at channel 225; fluorescence peak at channel 250).

lymphoma lines was verified by appropriate codominant expression of certain cell surface markers characteristic of the phenotypes of each of the respective partner cells, these early studies failed to demonstrate, where analyzed, expression of functional T cell characteristics (16, 24, 25). Subsequently, several laboratories have been successful in constructing T cell hybridomas expressing T cell function of the nonlymphoma partner as manifested by the active secretion of soluble mediators capable of exerting immunoregulatory effects (26–33); with one exception (27), these successful fusions have utilized the AKR T lymphoma line designated BW5147. All of these latter studies have been concerned with the construction of T cell hybridomas producing T cell factors capable of suppressing immune responses either in an antigen-specific manner for humoral responses (26, 27, 29–31) or delayed-type hypersensitivity responses (32), or in an antigen nonspecific manner for humoral responses (33) or selective for antibody responses of the IgE class (28).

A recent report (34) described the construction of a T hybridoma line capable of secreting biologically active factors effective in stimulating hematopoietic colony growth and B cell responses in vitro; however, this line did not secrete such factors spontaneously and required stimulation by concanavalin A to release active molecules into culture supernates. The present report constitutes, therefore, the first description of the successful construction of hybrid T cell lines spontaneously secreting a biologically active factor capable of enhancing immune responses, namely AEF.

The construction of AEF-secreting hybridomas was accomplished by fusing the BW5147 cell line with alloantigen-activated T cell blasts obtained after consecutive in vivo and in vitro activation steps. T cell blasts obtained in this manner yielded a relatively high fusion frequency with about one-third of such hybrids manifesting some degree of AEF-like biological activity. Two of the hybridomas secreting rather substantial quantities of AEF were chosen for the analyses presented herein. That these two lines secreting AEF, are indeed, true hybrids was verified by demonstrating codominant expression of the relevant allelic forms of Thy-1 antigens and the serological determinants of *H-2* contributed by each of the respective fused partner cells.

The supernates obtained from hybridomas 27 and 34 displayed typical functional characteristics ascribed to AEF. Thus, these hybridoma supernates were capable of activating B lymphocytes to respond to either SRBC or TNP-SRBC in cultures depleted of helper T lymphocytes normally required for such responses. Moreover, the typical T cell-activating properties of AEF, as described in recent studies from this laboratory (8–13), such as the ability to stimulate autonomous development of self-specific CTL and the capacity to directly stimulate T cell proliferation, were likewise present in these hybridoma supernates. The fact that the biological activities of AEF on both B and T lymphocyte targets coexisted in the same hybridoma supernates suggests, but does not prove, that these two distinct activities may be mediated by the same molecule; definitive proof on this issue must await complete cloning of these hybridoma lines and isolation to homogeneity of the active molecules secreted by them.

Evidence supporting the fact that the activities observed were, indeed, mediated by AEF, and not by one or more artifactual components of the cultured lines, can be summarized as follows: (*a*) supernates obtained for testing in the biological assays performed were free of all exogenous serum and/or protein constituents; (*b*) supernates

from the parent thymoma line cultured alone, as well as from numerous hybrids constructed from the same starting cell population, were devoid of any detectable AEF activity (moreover, such negative supernates were unable to suppress positive AEF-mediated biological effects, not shown); and (*c*) the AEF activities displayed by positive hybridomas, like those same activities exhibited by conventional preparations of AEF, could be abrogated by exposure to specific anti-Ia antibodies, thus verifying the expression of Ia determinants on the relevant molecules. It is interesting in the latter regard that most of the functional T cell hybridomas that have been successfully constructed, including those reported here, are lines capable of producing soluble mediators that bear *I* region determinants.

Another new finding described in the present report is the ability of these AEF-secreting hybridoma lines to respond to specific alloantigen stimulation. Responsiveness was verified by induced proliferation of hybridoma 34 after reexposure to fresh, irradiated $C3D2F_1$ target cells many months after initial fusion. This proliferative burst was accompanied by secretion of significantly increased quantities of AEF into supernates of such stimulated cultures, effective in exerting both B cell- and T cell-activating effects at a dilution factor well below detectable activities in supernates of unstimulated cultures of the same line. This contrasts with the failure of Köhler et al. (25) to observe either specific responsiveness or production of AEF in hybridomas constructed from MLR-stimulated T cells and the same BW5147 thymoma line. The differences between their results and ours reported here most likely relate to the degree of T cell activation used in obtaining the blasts for fusion. In their study, T cells obtained from primary MLR activation were employed, whereas in the present studies we utilized T cell blasts activated by consecutive in vivo and in vitro stages. Whereas our hybridoma is functionally responsive to specific antigen stimulation as evidenced by proliferation and increased secretion of AEF, it has not yet been determined whether our lines possess any specific cytolytic activities.

In addition to the results presented here, studies currently being conducted in collaboration with W. H. Fridman and colleagues have shown that both hybridomas 27 and 34 express Fc receptors (FcR) specific for immunoglobulins of the IgG class (FcRγ) (W. H. Fridman, et al. Manuscript in preparation.). Moreover, like a T cell hybridoma constructed by these same investigators that is also FcRγ positive (33), both hybridoma 27 and 34 secrete immunoglobulin-binding factor (IBF) activity (35–37), and their line (designated T2D4) appears to secrete AEF-like enhancing activity. It should be emphasized, however, that preferential expression of either AEF-enhancing or IBF-suppressive activities by the T2D4 and our hybridoma lines depends critically on the culture conditions used for the preparation of the biologically active supernates. Although exerting opposite, suppressive activity on T cell-dependent in vitro antibody responses, IBF, like AEF, has recently been found to bear Ia determinants (37). Details of these studies will be published elsewhere in the near future. It is interesting to find these two opposing biological activities, both of which are Ia positive, synthesized and secreted by the same T cell hybridoma lines, although the possible relevance of these findings will not be discussed here.

It is also worth noting one other point about the AEF-secreting hybridomas 27 and 34 lines, namely that the supernate of both lines is totally devoid of detectable interferon activity. In a recent extensive analysis of the possible contributions that interferon may make to the biological activities manifested by AEF preparations

obtained in the conventional manner, we have shown that removal of interferon contaminants from AEF by hydrophobic chromatography does not result in a corollary diminution of AEF activities, but rather tends to increase the expression of AEF effects (20). The fact that the AEF-secreting hybridomas described here are totally negative for interferon production proves beyond doubt that interferon is not responsible for any of the activities ascribed to AEF.

Finally, it is worth noting that because these T cell hybridomas secrete Ia-positive AEF molecules, our original contention that AEF is indeed derived from T cells (1, 4–6), a position contested somewhat by certain of the results of Delovitch et al. (38, 39), has been validated. Biochemical analysis will be undertaken once sufficient quantities of the cloned AEF-secreting hybridomas are available.

These AEF-secreting hybridoma lines, like others of a similar nature, should be invaluable for allowing future studies on the molecular biology of these important immunoregulatory molecules.

Summary

T cell hybridoma lines were constructed by fusion of DBA/2 alloantigen-activated T cell blasts with the AKR thymoma line BW5147. Certain of the hybridomas prepared in this manner secreted spontaneously into their culture supernates biologically active molecules that displayed B cell- and T cell-activating properties characteristic of allogeneic effect factor (AEF). Cell surface phenotype analysis documented that the hybridomas were, indeed, somatic cell hybrids between the two respective partner cells used for fusion. The B cell-activating properties of these hybridoma supernates was demonstrated by their capacity to stimulate T cell-depleted spleen cells to respond in vitro to T-dependent antigens. The T cell-activating properties of these hybridoma supernates was verified by their capacity to stimulate autonomous development of self-specific cytotoxic T lymphocytes and by their capacity to exert mitogenic effects on unprimed T cells. The biologically active molecules secreted by these hybridomas were, like conventional AEF, inhibitable by specific anti-Ia antibodies thus indicating the presence of Ia determinants on the relevant hybridoma products. Finally, these AEF-secreting hybridomas could be stimulated to proliferate and to secrete increased quantities of AEF when exposed to the specific alloantigen-bearing target cells to which the T cell blasts had been originally sensitized.

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References

1. Armerding, D., and D. H. Katz. 1974. Activation of T and B lymphocytes in vitro. II. Biological and biochemical properties of allogeneic effect factor (AEF) active in triggering specific B lymphocytes. *J. Exp. Med.* **140**:19.
2. Armerding, D., D. H. Sachs, and D. H. Katz. 1974. Activation of T and B lymphocytes in

vitro. III. Presence of Ia determinants on allogeneic effect factor (AEF). *J. Exp. Med.* **140:** 1717.

3. Armerding, D., R. T. Kubo, H. M. Grey, and D. H. Katz. 1975. Activation of T and B lymphocytes in vitro: presence of β_2-microglobulin determinants on allogeneic effect factor. *Proc. Natl. Acad. Sci. U. S. A.* **72:**4577.
4. Katz, D. H., D. Armerding, and Z. Eshhar. 1976. Histocompatibility gene products as mediators of lymphocyte interactions. *In* The Role of Products of the Histocompatibility Gene Complex in Immune Responses. D. H. Katz and B. Benacerraf, editors. Academic Press, Inc., New York. 541.
5. Katz, D. H. 1977. Lymphocyte Differentiation, Recognition and Regulation. Academic Press, Inc., New York. 598.
6. Eshhar, Z., D. Armerding, T. Waks, and D. H. Katz. 1977. Activation of T B. lymphocytes in vitro. V. Cellular locus, metabolism and genetics of induction and production of the allogeneic effect factor. *J. Immunol.* **119:**1457.
7. Armerding, D., Z. Eshhar, and D. H. Katz. 1977. Activation of T and B lymphocytes in vitro. VI. Biochemical and physiochemical characterization of the allogeneic effect factor. *J. Immunol.* **119:**1468.
8. Altman, A., T. E. Bechtold, J. M. Cardenas, and D. H. Katz. 1979. Biological effects of allogeneic effect factor on T lymphocytes: In vitro induction of cytotoxic T lymphocytes manifesting preferential lytic activity against *H-2* identical tumor cells. *Proc. Natl. Acad. Sci. U. S. A.* **76:**3477.
9. Altman, A., J. M. Cardenas, T. E. Bechtold, and D. H. Katz. 1980. The biological effects of allogeneic effect factor on T lymphocytes. I. The mitogenic activity and the autonomous induction of cytotoxic T lymphocytes by AEF. *J. Immunol.* **124:**105.
10. Altman, A., J. M. Cardenas, T. E. Bechtold, and D. H. Katz. 1980. The biological effects of allogeneic effect factor on T lymphocytes. II. The specificity of AEF-induced cytotoxic T lymphocytes. *J. Immunol.* **124:**114.
11. Altman, A., J. M. Cardenas, T. E. Bechtold, and D. H. Katz. 1980. The spontaneous induction of primary cytotoxic T lymphocyte responses in vitro by allogeneic effect factor. *In* Proceedings of the Second International Lymphokine Workshop. A. L. de Weck and M. Landy, editors. Academic Press, Inc., New York. 517.
12. Altman, A., and D. H. Katz. 1980. The induction of autoreactive T lymphocytes by allogeneic effect factor (AEF): relevance to normal pathways of lymphocyte differentiation. *Immunol. Rev.* **51:**3.
13. Altman, A., and D. H. Katz. Existence of T cells manifesting self-reactivity indistinguishable from alloreactivity. *J. Immunol.* In press.
14. Aarden, L. A. 1979. Revised nomenclature for antigen-nonspecific T cell proliferation and helper factors. *J. Immunol.* **123:**2928.
15. Aguet, M., L. C. Andersson, R. Andersson, E. Wight, H. Binz, and H. Wigzell. 1978. Induction of specific immune unresponsiveness with purified mixed leukocyte culture-activated T lymphoblasts as autoimmunogen. II. Analysis of the effects measured at the cellular and serological levels. *J. Exp. Med.* **147:**50.
16. Hammerling, G. J. 1977. T lymphocyte tissue culture lines produced by cell hybridization. *Eur. J. Immunol.* **7:**743.
17. Gefter, M. L., D. H. Margulies, and M. D. Scharff. 1977. Simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells. *Somatic Cell Genet.* **3:**231.
18. Click, R. E., L. Benck, and B. J. Alter. 1972. Immune responses in vitro. Culture conditions for antibody synthesis. *Cell. Immunol.* **3:**264.
19. Altman, A., T. E. Bechtold, and D. H. Katz. 1979. Manipulation of cytotoxic T lymphocyte responses to syngeneic tumors. I. Hapten-reactive helper T lymphocytes augment tumor-specific CTL responses in vitro. *J. Immunol.* **122:**2484.

20. Altman, A., J. M. Cardenas, R. M. Welsh, Jr. and D. H. Katz. 1980. The biological effects of allogeneic effect factor on T lymphocytes. III. Interferon does not contribute to the biological activities displayed by AEF on both T and B lymphocytes. *Ann. Immunol. (Paris).* **131**:335.
21. Katz, D. H., and D. P. Osborne, Jr. 1972. The allogeneic effect in inbred mice. II. Establishment of the cellular interactions required for enhancement of antibody production by the graft-versus-host reaction. *J. Exp. Med.* **136**:455.
22. Skidmore, B. J., and L. S. Miller. 1978. A new microcytotoxicity method for determining lymphoid chimerism by examination of peripheral blood lymphocytes of murine bone marrow chimeras. *J. Immunol. Methods.* **24**:337.
23. Köhler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond.).* **256**:495.
24. Goldsby, R. A., B. A. Osborne, E. Simpson, and L. A. Herzenberg. 1977. Hybrid cell lines with T-cell characteristics. *Nature (Lond.).* **267**:707.
25. Köhler, G., I. Lefkovits, B. Elliott, and A. Coutinho. 1977. Derivation of hybrids between a thymoma line and spleen cells activated in a mixed leukocyte reaction. *Eur. J. Immunol.* **7**: 758.
26. Kontiainen, S., E. Simpson, E. Bohrer, P. C. L. Beverley, L. A. Herzenberg, W. C. Fitzpatrick, P. Vogt, A. Torano, I. F. C. McKenzie, and M. Feldmann. 1978. T-cell lines producing antigen-specific suppressor factor. *Nature (Lond.).* **274**:477.
27. Taniguchi, M., and J. F. A. P. Miller. 1978. Specific suppressive factors produced by hybridomas derived from the fusion of enriched suppressor T cells and a T lymphoma cell lines. *J. Exp. Med.* **148**:373.
28. Watanabe, T., M. Kimoto, S. Maruyama, T. Kishimoto, and Y. Yamamura. 1978. Regulation of antibody response in different immunoglobulin classes. V. Establishment of T hybrid cell line secreting IgE class-specific suppressor factor. *J. Immunol.* **121**:2113.
29. Taussig, M. J., J. R. F. Corvalan, R. M. Binns, and A. Holliman. 1979. Production of an H-2-related suppressor factor by a hybrid T-cell line. *Nature (Lond.).* **277**:305.
30. Taussig, M. J., and A. Holliman. 1979. Structure of an antigen-specific suppressor factor produced by a hybrid T-cell line. *Nature (Lond.).* **277**:308.
31. Taniguchi, M., T. Saito, and T. Tada. 1979. Antigen-specific suppressive factor produced by a transplantable I-J bearing T-cell hybridoma. *Nature (Lond.).* **278**:555.
32. Hewitt, J., and F. Y. Liew. 1979. Antigen-specific suppressor factors produced by T cell hybridomas for delayed-type hypersensitivity. *Eur. J. Immunol.* **9**:572.
33. Neauport-Sautes, C., C. Rabourdin-Combe, and W. H. Fridman. 1979. T-cell hybrids bear Fcγ receptors and secrete suppressor immunoglobulin binding factor. *Nature (Lond.).* **277**: 656.
34. Schrader, J. W., B. Arnold, and I. Clark-Lewis. 1980. A con A-stimulated T-cell hybridoma releases factors affecting haematopoietic colony-forming cells and B-cell antibody responses. *Nature (Lond.).* **283**:197.
35. Fridman, W. H., and P. Goldstein. 1974. Immunoglobulin-binding factor present on and produced by thymus-processed lymphocytes (T cells). *Cell. Immunol.* **11**:442.
36. Fridman, W. H., R. H. Gisler, A. Guimezanes, and C. Neauport-Sautes. 1976. Suppression of in vitro antibody synthesis by a T cell product. Its relation with the Fc receptor of activated T cells. *In* Leukocyte Membrane Determinants Regulating Immune Reactivity. V. P. Eijsvoogel, D. Roos, and W. P. Zeijlemaker, editors. Academic Press, Inc., New York. 331.
37. Rabourdin-Combe, C., M. E. Dorf, A. Guimezanes, and W. H. Fridman. 1979. T cell-produced immunoglobulin binding factor (IBF) bears determinants coded for by the *I*-region of the major histocompatibility complex and lacks allogeneic restriction. *Eur. J. Immunol.* **9**:237.

38. Delovitch, T. L., and H. O. McDevitt. 1977. In vitro analysis of allogenic lymphocyte interaction. I. Characterization and cellular origin of an Ia-positive helper factor-allogeneic effect factor. *J. Exp. Med.* **146:**1019.

39. Delovitch, T. L., J. Biggin, and F.-Y. Fung. 1978. In vitro analysis of allogeneic lymphocyte interaction. II. *I*-region control of the activity of a B-cell-derived *H-2*-restricted allogeneic effect factor and its receptor during B-cell activation. *J. Exp. Med.* **147:**1198.