Ia ANTIGEN-BEARING B CELL TUMOR LINES CAN PRESENT PROTEIN ANTIGEN AND ALLOANTIGEN IN A MAJOR HISTOCOMPATIBILITY COMPLEX-RESTRICTED FASHION TO ANTIGEN-REACTIVE T CELLS

By LAURIE H. GLIMCHER, KYUNG-JIN KIM, IRA GREEN, and WILLIAM E. PAUL

From the Laboratory of Immunology, and the Laboratory of Microbial Immunity, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

It is clear that the activation of T lymphocytes for helper (1–3), cytotoxic (4), delayed type hypersensitivity (5), and antigen-induced proliferative (6, 7) responses requires their interaction with a specialized antigen-presenting cell (APC)[1] that expresses I-region-associated (Ia) antigens. Cells of the monocyte-macrophage lineage (8, 9), including the liver Kupffer cell (10) and the Langerhan's cell of the epidermis (11) as well as the recently described dendritic cell (12), have all been shown to be effective APC and in addition to serve as stimulator cells in mixed lymphocyte reactions (13). Antigen-specific T cells appear to recognize a complex of antigen and Ia molecules on the surface of such an APC, and such "joint recognition" is responsible for the phenomenon of major histocompatibility complex (MHC) restriction. However, the biochemical nature of the antigen processing event and the sequence of the T cell-APC signals that result in a T cell response have remained difficult to dissect largely because of technical problems in obtaining sufficiently large numbers of highly purified APC.

A tumor cell line that possesses APC ability would clearly be an extremely useful tool; however, the macrophage-like tumor lines characterized thus far appear to have little or no surface Ia antigen and are largely deficient in APC ability (14). Recently, Chesnut and Grey (15) have presented data that normal B cells under certain circumstances can present antigen to T cells. Indeed, Schwartz et al. (16) and Chesnut and Grey (personal communication) have presented preliminary data that an Ia antigen-bearing B cell tumor line could serve as an APC for antigen-reactive T cells. Certainly it appears that the B cell must be capable of presenting the antigen-Ia complex to T lymphocytes because cloned lines of T lymphocytes can interact in an antigen-specific, MHC-restricted fashion with B cells after initial activation by marophages (17).

In this paper, we report that several but not all of a series of Ia antigen-positive B cell tumor lines are capable of presenting both alloantigens and protein antigens to

[1] *Abbreviations used in this paper:* APC, antigen presenting cell; C, complement; CFA, complete Freund's adjuvant; GLϕ, poly(Glu^{57}Lys^{35}Phe^{8}); Ia, I region associated; IL-1, interleukin 1; IL-2, interleukin 2; LN, lymph node; MHC, major histocompatibility complex; MLR, mixed lymphocyte response; OVA, ovalbumin; PMA, phorbol myristic acetate; PPD, purified protein derivative of tuberculin; SAC, splenic adherent cells.

alloreactive and antigen-reactive T lymphocytes in an MHC-restricted fashion. The addition of interleukin 1 (IL-1) is necessary for the stimulation of allogeneic responses and, although not necessary, did potentiate antigen-specific responses. The activation of alloreactive T cells is blocked by the inclusion in the cultures of specific monoclonal anti-Ia antibodies. Furthermore, it is possible to obtain an antigen-specific response by pulsing the tumor cells with antigen, although the continuous presence of soluble antigen in the culture system results in a superior response. These tumor cells, by comparison with conventional APC, should prove useful in studying the biochemical events that occur during antigen processing and the requirements for T cell triggering by processed antigen in association with Ia molecules.

Materials and Methods

Animals. B10.A, C57BL/6, and (C57BL/6 × BALB/c)F_1 [(B × C)F_1] strains were purchased from The Jackson Laboratory, Bar Harbor, Maine. The BALB/c AnN, C3H/HeN, and B10.D2/SnN strains were obtained from the Division of Research Services, National Institutes of Health. The B10.M/Sg strain was bred in our animal colony from breeding pairs kindly sent by Dr. Jack Stimpfling, Great Falls, Mont.

Tumor Cells. The six BALB/c B lymphoma cell lines (A20.3, M12.4, K46R.18, X16C8.4, L10A6.2, and BALENLM 17.7.2) have been previously characterized (18), as has the T cell lymphoma line (BALENTL 3) (19), and were grown in a culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) supplemented with 8% fetal calf serum, 15 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, and L-glutamine (300 μg/ml). The phenotypic characteristics of each of these cell lines is summarized in Table III.

Antigens and Immunizations. Purified protein derivative of *Mycobacterium tuberculosis* (PPD) (Connaught Medical Research Laboratory, Willowdale, Ontario) was used in culture at 20 μg/ml. Pigeon cytochrome *c* (Sigma Chemical Co., St. Louis, Mo.) was used in culture at 100 μg/ml. The random terpolymer poly(Glu^{57}Lys^{35}Phe^{8}) (GLϕ^{8}) was purchased from Miles-Yeda (Rehovot, Israel) and was used at 50 μg/mouse for immunization and at 50 μg/ml in culture. Calf skin collagen (Sigma Chemical Co.) was the kind gift of Dr. Stephen Hedrick, (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases) and was used at 100 μg/mouse for immunization and at 100 μg/ml in culture. Ovalbumin (OVA) purchased from Miles-Yeda was used in culture at 100 μg/ml. All immunizations were carried out in the hind footpads by injecting 0.1 ml of an emulsion that contained a 1:1 mix of antigen in normal saline and complete Freund's adjuvant (CFA) that contained 1 mg/ml of *M. tuberculosis* strain H37Ra.

Antisera. A.TH anti-A.TL (αIa) alloantisera (an antiserum made by immunizing A.TH mice with A.TL cells) and the monoclonal reagent 14-4-4S (α*I-E*) were the kind gifts of Dr. D. H. Sachs and Dr. Keiko Ozato, National Cancer Institute. The monoclonal anti-$I\text{-}A^k$ reagent (clone 10.2.16), reactive with the public specificity Ia.17, was kindly provided by Dr. Richard Hodes, National Cancer Institute. The anti-$I\text{-}A^d$ reagent (clone MKD6) was derived by Dr. P. Marrack and Dr. J. Kappler (National Jewish Hospital, Denver, Co.) and was the kind gift of Dr. J. Berzofsky, National Cancer Institute. The MKD6 and the 10.2.16 monoclonal reagents were culture supernatants and were used at a 1:15 dilution in the culture system for the blocking studies shown in Table II. The 14-4-4S was in ascites form and was used at 1:200 dilution for the blocking studies. Monoclonal rat IgG_{2a} anti-ThB antibody (17C9), which reacts with B cells and thymocytes, was purified from hybridoma culture supernatants by affinity chromatography using a monoclonal mouse anti-rat $IgG_{1,2a}$ column and was the kind gift of Dr. John Kung (NIAID).

Antigen-specific T Cell Proliferation Assay. T cells were purified from draining lymph nodes (LN) by passage over nylon wool columns, as previously described (7). The nylon-passed cells were then treated with A.TH anti-A.TL alloantiserum and complement to further deplete contaminating Ia antigen-positive macrophages. Each 10^7 cells was treated for 30 min with 1 ml of a 1:50 dilution of the antiserum, washed, and then treated for 35 min at 37° with 1 ml

of a 1:20 dilution of rabbit complement (C; Lo-Tox, Cedarlane Laboratories Limited, Hornby, Ontario, Canada). These nylon-passed αIa + C-treated T cells were cultured in RPMI 1640 containing L-glutamine (300 ug/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (5×10^{-5}M), Hepes buffer (10 mM), and 2.5% human AB serum (obtained from a single donor). Cells (2.5×10^5 cells/0.2 ml) were cultured in flat-bottomed microtiter plates together with 1×10^5 to 2×10^5 spleen cells or varying numbers of tumor cells, with or without antigen. In some instances, APC were pulsed with antigen before addition to cultures. These antigen-pulsed presenting cells were prepared by incubating tumor or spleen cells in PPD 100 μg/ml for 1 h at 37° and washing five times in media.

Spleen cells used as APC received 2,000 rad, and tumor cells received 10,000 rad, using a 137 Cs irradiator. In all experiments the A20.3 tumor cells were also cultured alone. In addition, in some experiments IL-1 and antigen were added to cultures containing tumor cells alone; the cpm obtained did not vary whether IL-1 or antigen was present or absent. Examination of stimulated cultures containing T cells and A20.3 at day 4 revealed that cells with the morphology of tumor cells were in a state of degeneration, whereas the blasts present had the typical appearance of T lymphocytes. Stimulation was assessed at day 4 by measuring the incorporation of [^{3}H]thymidine (1 μCi/well) (New England Nuclear, Boston, Mass.) into macromolecules. Data are expressed as the arithmetic mean cpm. SE of the mean of the replicates was usually <10% of the mean counts and has been omitted from the tables for simplicity.

Allogeneic Mixed Lymphocyte Response (MLR). Spleen or lymph node cells from unprimed mice were passed over nylon wool columns and treated with αIa + C. These cells (3×10^5) were cocultured with irradiated spleen cells or tumor cells, as stimulator cells, in round-bottomed microtiter wells in 0.2 ml of media, as described above. The response was assayed on day 4 by uptake of [^{3}H]thymidine.

Antigen-reactive and Alloreactive T Cell Lines. The two antigen-reactive T cell lines (a BALB/c OVA-specific line and a B10.A pigeon cytochrome *c*-specific line) were the kind gifts of Dr. Maureen Howard and Dr. Louis Matis, respectively (Laboratory of Immunology, NIAID) and were prepared by the technique of Kimoto and Fathman (20). The T cells were maintained as long-term continuous cultures by alternating 4-d periods of restimulation with antigen and syngeneic irradiated (3,300 rad) spleen cells with 10- to 14-d periods without antigen. Antigen-reactive T cells recovered at the end of a 14-d rest period were used to assay specific antigen presentation by spleen or tumor antigen-presenting cells. Two alloreactive lines, D3 and M15, were prepared from original soft agar colonies by the technique of Sredni et al. (21) and were the kind gift of Dr. Ronald Schwartz and Mrs. Chuan Chen (Laboratory of Immunology, NIAID). They are of B10.A origin and are specific for DNP-OVA presented by B10.A APC. In addition, line D3 has allospecificity for the H-2^d haplotype, and line M15 has allospecificity for the H-2^f haplotype. The lines were assayed in the following way. T cells (1×10^4 to 2×10^4) were cocultured with 1×10^5 to 5×10^5 irradiated spleen cells or varying numbers of irradiated tumor cells in the presence or absence of antigen in flat-bottomed microtiter wells in the complete media described above, with the exception that 10% fetal calf serum was substituted for human serum. [^{3}H]thymidine uptake was assayed on days 3–4. Lines D3 and M15 required the presence of an IL-2-containing EL4 culture supernatant during the culture period.

IL-1 Preparations. A P388D_1 culture supernatant containing IL-1 was the kind gift of Dr. Maureen Howard and Mrs. Barbara Johnson (Laboratory of Immunology, NIAID) and was produced according to the method of Mizel and Mizel (22). Briefly, the P388D1 macrophage-like cell line was incubated with 1 μg/ml of phorbol myristate acetate (PMA) in media containing 1% fetal calf serum. At 5 d the supernatant was harvested and incubated twice with inactivated charcoal to remove PMA. Labeling such supernatants with radiolabeled PMA (New England Nuclear) indicated that charcoal absorption results in removal of >90% of PMA (A. L. DeFranco, unpublished observations). The absorbed supernatant was then concentrated sevenfold by vacuum dialysis. This preparation contained IL-1 activity, as assayed by induction of proliferation of C3H/HeJ thymocytes in the presence of concanavalin A. A maximum induction of proliferation was seen at a dilution of supernatant of 1:16. In most experiments, unless otherwise indicated, this preparation was used at a final concentration of 1.25% in the

culture system. A highly purified preparation of murine IL-1 was the kind gift of Dr. Steven Mizel, Pennsylvania State University, State College, Pa., and has been recently described (23).

Results

A20.3, an Ia Antigen-positive B Cell Tumor, Can Stimulate an Allogeneic MLR in the Presence of IL-1. Spleen cells that have been enriched for B lymphocytes and depleted of splenic adherent cells (SAC) by various maneuvers have been found to be ineffective in eliciting allogeneic T cell proliferation (13). Because both SAC and B cells have surface Ia molecules, one possibility was that the failure of the B cell population to stimulate was due to the lack of some secretory product possessed by authentic stimulator cells and necessary for eliciting alloreactive T cell proliferation. A very likely candidate seemed to be Il-1 (24). Indeed, Table I demonstrates that in the presence of a small amount of an Il-1-rich P388D$_1$ supernatant, an excellent allogeneic MLR was obtained using 10^4 to 10^5 cells from A20.3, an Ia antigen-positive BALB/c B cell tumor line, as stimulator cells. No significant allogeneic MLR was seen in the absence of added supernatant. Moreover, the A20.3 tumor cells did not elicit syngeneic proliferation, ruling out a nonspecific stimulatory effect of tumor or viral antigens or other immunostimulatory secretory products.

TABLE I

An Ia-positive B Cell Tumor Can Stimulate an Allogeneic MLR in the Presence of IL-1

Experiment	Stimulator cell	[^{3}H]Thymidine uptake of responder cell population*			
		Unfractionated B6 spleen		Unfractionated BALB/c spleen	
		Without IL-1	With IL-1‡	Without IL-1	With IL-1
		Δ*cpm*			
1	None	26,092	15,931	17,236	13,372
	BALB/c spleen (4×10^5)	70,916	151,667	11,112	10,451
	A20.3 (10^5)§	22,895	262,540	2,235	5,559
	A20.3 (10^4)	33,135	59,028	16,840	14,280
		Nylon-passed αIa + C-treated B6 spleen			
		Without IL-1		With IL-1	
2	None	162		1,227	
	BALB/c spleen (4×10^5)	37,068		109,993	
	A20.3 (10^5)§	1,631		234,106	
	A20.3 (10^4)	0		64,684	

* 3×10^5 responder cells were cocultured with the indicated stimulator cells for 3 d at 37°C in round-bottomed microtiter wells. Cultures were pulsed with 1μCi of [^{3}H]thymidine 16 h before harvesting.

§ A20.3 tumor cells were irradiated with 10,000 rad using a 137 Cs irradiator. The proliferation of irradiated A20.3 cells by themselves was 28,182 cpm (10^5) and 8097 cpm (10^4) in experiment 1 and 11,548 cpm (10^5) and 1,942 cpm (10^4) in experiment 2. These numbers have been subtracted from the cpm shown.

‡ IL-1-containing supernatant from PMA-stimulated P388D$_1$ cells was included at a final concentration of 1.25%.

The responder cell population in experiment 1 was unfractionated spleen cells. To rule out the possibility that Ia alloantigens were shed from A20 tumor cells and then taken up, reprocessed, and re-presented to the T cells by syngeneic macrophages in the responder cell population, two approaches were taken. First, as shown in Table I, experiment 2, the responder cell population was passed over nylon wool columns and then treated with an α-Ia antiserum and C to further deplete Ia antigen-bearing macrophages. The allogeneic MLR obtained using this population as responder cells was as good as that obtained using unfractionated spleen cells as the responder population. Second, blocking studies using monoclonal antibodies directed at Ia antigens present on either the responder or stimulator cell population were performed and demonstrated that antibodies directed at Ia antigens present on A20.3 stimulator cells inhibited the allogeneic MLR. Table II shows that the allogeneic MLR produced by coculturing A20.3 stimulator cells with C3H(H-2^k) responder cells was blocked 62–69% by the inclusion of the anti-I-A^d reagent (MKD6) in the culture system, 52–69% by the inclusion of the anti-I-E antibody (14-4-4S) in the culture supernatant, and 86% when both reagents were included in the culture system. The anti-I-A^k reagent (10.2.16), which is directed at the I region determinants of the responder cell population, and the ThB reagent, which is directed against a determinant present on B cells and thymocytes, caused no significant inhibition when they were included in the culture system. The allogeneic MLR produced by control BALB/c spleen cells was affected similarly by the above reagents. These same reagents did not significantly inhibit, or even enhanced, an allogeneic MLR elicited by B6(H-2^b) stimulator cells.

Not All BALB/c B Cell Tumors Can Stimulate an Allogeneic MLR. A number of BALB/c B cell tumors have been screened for the expression of and density of surface Ia antigens (K. J. Kim, manuscript in preparation). These tumor cell lines were

TABLE II

*Effect of Monoclonal Anti-Ia Antibodies on the Allogeneic MLR Stimulated by A20.3 cells**

Stimulator cell	Antibody specificity	Experiment 1		Experiment 2		Experiment 3		
		[^{3}H]Thymidine uptake	Percent inhibition	[^{3}H]Thymidine uptake	Percent inhibition	Antibody specificity	[^{3}H]Thymidine uptake	Percent inhibition
A20.3‡	None	175,391 ± 16,719	—	13,571 ± 2,144		None	38,168 ± 3.752	—
	I-A^d	66,843 ± 7,211	62	4,150 ± 300	69	ThB	30,835 ± 2,478	19
	I-E	54,378 ± 7,151	69	6,577 ± 505	52	I-A^d + I-E	11,842 ± 758	69
	I-A^k	134,315 ± 10,435	24	16,434 ± 3,085	−21			
	I-A^d + I-E	ND§		1,909 ± 748	86			
BALB/c	None	189,299 ± 233		147,625 ± 15,357		None	93,969 ± 5,544	
	I-A^D	58,133 ± 166	69	66,475 ± 2,000	55	ThB	69,794 ± 2,707	26
	I-E	116,138 ± 9,324	39	66,714 ± 6,695	55	I-A^d + I-E	4,934 ± 1,643	95
	I-A^k	170,466 ± 600	10	106,002 ± 13,519	28			
	I-A^d + I-E	ND		27,808 ± 4,136	81			
B6 spleen	None	215,033 ± 10,336		156,505 ± 9,464				
	I-A^d	212,894 ± 22,738	1	155,121 ± 15,346	1			
	I-E	217,655 ± 6,990	−1	159,317 ± 26,617	−1			
	I-A^k	285,516 ± 16,413	−33	165,353 ± 17,743	−11			
	I-A^d + I-E	ND		168,191 ± 16,736	−11			

* In the first two experiments, the responder cells were nylon-column passed, αIa + C-treated C3H spleen cells, and in the third experiment, the responder cells were of C57BL/6 origin. The monoclonal reagents, MKD6, directed against a determinant in the *I-A^d* subregion, and 10.2.16, directed against a determinant in the *I-A* subregion of the k haplotype, were included at a dilution of 1:15 (vol/vol) in the culture system. The monoclonal reagent 14-4-4S, directed against a determinant in the *I-E* subregion (specificity Ia7), was in ascites form and was included at a dilution of 1:200 in the culture system. The monoclonal reagent ThB was affinity-purified hybridoma supernatant and was used at 1 μg/ml final concentration. All three experiments were performed in the presence of 1.25% IL-1.

‡ In experiments 1 and 3 there were 10^5 A20.3 cells per well, and in experiment 2 there were 10^4 A20.3 cells per well.

§ Not done.

therefore assayed for their ability to stimulate an allogeneic MLR. The phenotypes of these tumors is shown in Table III. Surprisingly, as shown in Table III, only three (A20.3, M12.4, and X16C8.4) of the six tumors were able to stimulate allogeneic T cell proliferation; the three remaining tumors (K46R.18, L10A6.2, and BALENLM 17.7.2) were ineffective stimulators despite the possession of large amounts of surface Ia. There was no obvious correlation between the amount of surface Ia expressed on a tumor line as determined by fluorescence activated cell sorter analysis and its stimulatory ability (data not shown). Thus, the M12 and X16C tumor cells have relatively little surface Ia but were powerful stimulants, whereas the K46, L10A, and BALENLM 17 have large amounts of surface Ia and were poor stimulators.

A20.3 Stimulates Proliferation by an H-2^d but Not by an H-2^f Alloantigen-specific Line. Further proof that A20.3 tumor cells are themselves providing the alloantigenic stimulus for T cell proliferation and not simply providing a nonspecific stimulus or causing the differentiation or activation of APC in residual spleen cells was obtained by using two long-term alloreactive B10.A T cell lines. One of these lines, D3, was specific for an alloantigen of the H-2^d haplotype and the other, M15, was directed at an alloantigen of the H-2^f haplotype. The results in Table IV show that as few as 5 × 10^3 A20.3 cells induced proliferation by the D3 line of a magnitude greater than that elicited by 10^5 control BALB/c spleen cells. By contrast, the A20.3 cells were ineffective in stimulating the M15 line; this line could, however, be stimulated by B10.M (H-2^f) spleen cells. Unlike the results obtained using whole populations of T cells, proliferation of the cloned T cells could be elicited in the absence of exogenous IL-1. The reason for this discrepancy is unclear; however, these assays were performed in the presence of a preparation of IL-2.

A20.3 Presents Antigen to In Vivo Primed T Cells. An Ia antigen-bearing APC appears to be the cell responsible for stimulating both an allogeneic MLR and for presenting

TABLE III

*Not All BALB/c B Cell Tumors Can Stimulate an Allogeneic MLR**

Designation	Stimulator cell surface markers‡				[^{3}H]Thymidine uptake of responder cell	
	Ig	IgM	IgG	Ia§	Allogeneic (B6) T cells	Syngeneic (B10.D2) T cells
					Δ*cpm*	
None					798	1,025
B10.D2 spleen					47,578	11,408
A20.3	+	−	±	+++	41,258	8,049
M12.4	±	−	−	+	44,560	3,050
X16C 8.4	+	+	−	+	66,280	9,338
K46R.18	+	+	−	++	3,056	338
BALENLM17.7.2	+	+	−	++	3,215	797
L10A6.2	+	+	±	++	803	646

* 3 × 10^5 nylon-passed αIa + C-treated B6 or B10.D2 spleen responder cells were cocultured for 3 d with different B cell tumors (10^4 cells/well) or with control spleen cells in the presence of 1.25% IL-1.

‡ All of these tumors were negative for C3R and positive for FcR.

§ The presence of Ia antigens on these tumor cell lines has been established by immunofluorescence and cytotoxicity with αIa alloantisera (see ref. 18) and more recently has been confirmed with monoclonal anti-Ia reagents on a fluorescence-activated cell sorter (Kim et al., manuscript in preparation).

TABLE IV

*A20.3 Stimulates Only an H-2^d and Not an H-2^f Alloantigen-specific Line**

Il-1	Stimulator cells	[^{3}H]Thymidine uptake		
		Line D3		Line M15
		Experiment 1	Experiment 2	
			Δcpm	
None	None	594	1,833	190
	BALB/c spleen (10^5)	2,274	11,919	—
	B10.M (H-2^f) spleen (10^5)	—‡	—	2,380
	A20.3 (10^4)	12,279	28,030	0
	A20.3 (5×10^3)	18,639	—	469
	A20.3 (10^3)	975	—	162
1.25%	None	325		289
	BALB/c spleen (10^5)	1,976		—
	B10.M (H-2^f) spleen (10^5)	—		4,669
	A20.3 (10^4)	24,973		0
	A20.3 (5×10^3)	14,995		611
	A20.3 (10^3)	1,789		248

* Lines D3 and M15 are of B10.A origin and are both specific for DNP-OVA. Both lines also have an alloreactive specificity. D3 has anti-H-2^d alloreactivity, and M15 has anti-H-2^f alloreactivity. In these experiments, 2×10^4 D3 cells and 1×10^4 M15 cells were cocultured with varying numbers (indicated in parentheses) of irradiated A20.3 cells or control spleen cells in the presence of 2% IL-2. The addition of IL-2 was necessary because these lines are IL-2-dependent. Proliferation was assayed on day 3.

‡ Not done.

antigen to antigen-reactive T cells. Table V demonstrates that a small number (10^3 to 10^4) of A20.3 cells are capable of presenting three different antigens—OVA, PPD, and GLϕ—to T cells from primed donors. Indeed, 10^3 A20.3 cells stimulated a response that was slightly greater than that stimulated by 10^5 B10.D2 spleen cells. As in the experiments with alloreactive T cells, the antigen-reactive T cell population has been rigorously depleted of Ia-positive cells by nylon column passage followed by treatment with an anti-Ia antiserum + C. The antigen-specific response of this T cell population to antigen in the absence of added antigen-presenting cells was minimal as shown, whereas nylon column passage alone without such treatment results in a very substantial antigen-specific response (not shown).

The experiments presented in Table V were performed in the presence of 1.25% of the IL-1-rich P388D1 PMA-stimulated supernatant. However, unlike the results obtained in the allogeneic MLR (Tables I, II, and III), it was not necessary to add exogenous IL-1 to the culture system to obtain a good antigen-specific response. As shown in Table VI, 5×10^3 A20.3 cells stimulated a good T cell response to antigen in the absence of added IL-1. This response was, however, clearly magnified by the addition of small amounts of either crude IL-1 or of highly purified IL-1. The mechanism of the enhancement of the proliferation by exogenous IL-1 is unclear; incubation of A20.3 cells with this preparation does not increase the density of surface Ia (K. J. Kim, manuscript in preparation).

Antigen Pulsing of A20.3 Cells. Previous studies have demonstrated that antigen-specific T cell proliferation can be obtained if the antigen is present continuously

TABLE V

A20.3, M12.4, and X16C8.4 Present Antigen to T Cells from Primed Donors

	[^{3}H]Thymidine uptake*				
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
	OVA response	PPD response	GLϕ response	PPD response	PPD response
			Δcpm		
T alone	2,578	194	495	241	1,308
T + B10.D2 spleen‡					
2×10^5	41,988	50,297	18,465	11,429	47,241
1×10^5	28,077	—§	—	5,934	
10^4	8,726	—	—		
10^3	4,922	—	—		
T + A20.3					
10^5	130,965	126,042	44,368	54,725	39,707
10^4	73,469	142,293	60,825		
10^3	28,821	28,385	—		
T + X16C8.4 10^5	—	—	—	13,855	—
T + M12.4 10^5	—	—	—	—	39,727

* 2.5×10^5 nylon column-passed αIa + C-treated cells from B10.D2 mice primed with OVA or saline in CFA or from (B × C)F_1 mice primed with GLϕ in CFA in the footpads 8 d earlier were cocultured with irradiated spleen cells or varying numbers of A20.3 cells in the presence of IL-1 (1.25%) with or without antigen (PPD 20 μg/ml, GLϕ 50 μg/ml, and OVA 100 μg/ml). Proliferation was assessed on day 4. Results are expressed as cpm obtained in the presence of antigen minus cpm obtained without antigen.

‡ (B × C)F_1 spleen in experiment 3.

§ Not done.

TABLE VI

*Effect of Exogeneous IL-1 on Antigen Presentation by A20.3 to T Cells from Primed Donors**

Experiment 1	PPD-stimulated [^{3}H]thymidine uptake		
	Without IL-1	IL-1 (1.25%)	Purified IL-1 (5 U/ml)
		Δcpm	
T alone	3,351	11,398	6,750
T + B10.D2 spleen (2×10^5)	70,447	99,884	111,215
T + A20.3 (10^5)	57,262	65,596	82,265
T + A20.3 (10^4)	57,161	92,521	91,458
T + A20 (5×10^3)	35,302	68,334	89,799
Experiment 2			
T alone	238	691	
T + B10.D2 spleen (2×10^5)	259,626	221,158	
T + A20.3 (10^5)	20,993	73,667	
T + A20.3 (10^4)	56,454	170,516	

* Experimental conditions are the same as described in the footnote to Table V, with the exception that cultures were done either (*a*) in the absence of exogenous IL-1, (*b*) in the presence of 1.25% (vol/vol) of an IL-1-containing supernatant made by stimulating P388D_1 cells with PMA, or (*c*) in the presence of a highly purified preparation of IL-1 kindly donated by Dr. Steven Mizel and recently described (23).

during the culture period or if the APC are first pulsed with the antigen, washed to remove unbound antigen, and then cocultured with the antigen-reactive T cells (25). To determine whether A20.3 cells could be effectively pulsed with antigen or if they required the continuous presence of antigen to stimulate an antigen-specific T cell response, we incubated A20.3 cells and control spleen cells with PPD (100 μg/ml) for 1 h. The cells were then washed extensively (five times) to remove unbound antigen and added to PPD-reactive T cells. Table VII shows that 10^5 pulsed A20.3 cells induced a T cell proliferative response that was 50–90% as good as the response generated when the antigen was present in the culture medium throughout the assay period. Pulsed control spleen cells were 40–70% as effective as spleen cells plus continuous antigen in this experiment. However, when $<10^5$ pulsed A20.3 cells were used in culture, no response was stimulated. In contrast, as few as 10^3 tumor cells permitted a good response (40,123 cpm) in the presence of continuous antigen. Again, the proliferation obtained was magnified by the addition of the IL-1 preparation to the culture system.

A20.3 Presents Antigen to In Vivo Primed T Cells and to Long-Term Antigen-reactive T Cell Lines in a MHC-restricted Fashion. In physiological situations, antigen-specific proliferative responses are only obtained when the antigen-reactive T cells and the APC are derived from donors that share common I region alleles (6, 7). The antigen-specific T cell appears to recognize both an antigenic determinant and a syngeneic I region-encoded product on the surface of the APC. To determine whether A20.3 cells also presented antigen in such an MHC-restricted fashion, two different approaches were taken. First, *Ir* gene controlled systems were investigated. In such systems, it is known that for the APC to be able to present a given antigen, the donor of the APC must possess a responder allele in the I region (26). A20.3 cells were tested for their ability to present antigen to T cells from (C57BL/6 × BALB/c)F_1 mice primed with antigens to which one parent was a responder and the other parent a nonresponder. C57BL/6 mice possess an Ir gene enabling them to respond to collagen (27, 28) but are

TABLE VII

*Presentation of Antigen by Pulsed A20.3 Cells**

	[^{3}H]Thymidine uptake					
	Without IL-1		IL-1 (1.25%)		Purified IL-1 (4 U/ml)‡	
	Continuous antigen	Pulsed	Continuous antigen	Pulsed	Continuous antigen	Pulsed
	Δ*cpm*					
T alone	494	—	376	—	520	—
T + B10.D2 spl.	99,651	59,366	108,304	39,450	106,292	78,081
T + A20.3 10^5	52,927	38,564	78,437	39,774	67,307	59,031
T + A20.3 10^4	51,995	0	108,053	0	46,307	0
T + A20.3 5×10^3	28,228	0	104,379	0	67,046	0
T + A20.3 10^3	2,488	0	40,123	0	12,519	0

* 2.5×10^5 nylon-passed αIa + C-treated CFA-primed LN cells from a B10.D2 animal were cocultured with varying numbers of irradiated A20.3 cells or 2×10^5 spleen cells in the presence of continuous antigen (PPD, 20 μg/ml) or with A20.3 and B10.D2 spleen cells that had been pulsed with 100 μg/ml of PPD for 1 h and extensively washed. The cpm obtained in the absence of antigen has been subtracted from the numbers shown above.

‡ See footnote to Table VI.

nonresponders to GLϕ; BALB/c mice have the reverse response pattern; they respond to GLϕ but not to collagen. Both parental strains respond to PPD. In this experiment (Table VIII), F_1 spleen cells were capable of presenting each of the three antigens to T cells from primed F_1 donors. A20.3 cells which are of H-2^d haplotype, only stimulated F_1 T cell responses to GLϕ and PPD. They did not stimulate responses to collagen to which mice of the H-2^d haplotype are nonresponders. This experiment indicates that antigen presentation by A20.3 is regulated by Ir gene products and thus strongly argues against the possibility that the A20.3 serves only to induce the

TABLE VIII

A20.3 Presents Antigen in a Genetically Restricted Fashion to T Cells from a Primed Donor

	[^{3}H]Thymidine uptake*		
	GLϕ	Collagen	PPD
		Δ*cpm*	
T alone	41	677	289
T + (B × C)F_1 spleen	10,884	16,605	53,287
T + A20.3 (10^5)	18,127	0	89,439
T + A20.3 (10^4)	14,884	788	113,616
T + A20.3 (5×10^3)	12,600	0	75,234

* 2.5×10^6 nylon-passed αIa + C-treated LN T cells from a (B × C)F_1 animal primed 10 d previously with 100 μg GLϕ and 100 μg of collagen in CFA were cocultured for 4 d with either 2×10^5 (B × C)F_1 spleen cells or varying numbers of A20.3 cells in the presence of 1.25% IL-1. Results are expressed as the cpm obtained in the presence of antigen minus the cpm obtained with media alone.

TABLE IX

*A20.3 Presents Antigen in a Genetically Restricted Fashion to Long-Term T Cell Lines**

Stimulator cell	[^{3}H]Thymidine uptake			
	Without IL-1		IL-1 1.25%	
	BALB/c OVA specific line	B10.A pigeon cytochrome *c*-specific line	BALB/c OVA-specific line	B10.A pigeon cytochrome *c*-specific line
		Δ*cpm*		
None	46	152	179	62
B10.D2 spleen	55,457	114	87,748	93
B10.A spleen	ND‡	57,934	ND	70,643
A20.3 10^4	56,489	3,119	69,167	305
A20.3 5×10^3	60,122	ND	89,298	ND
A20.3 10^3	30,416	0	71,826	557

* 2×10^4 T cells from a BALB/c OVA-specific T helper line or 1×10^4 T cells from a B10.A pigeon cytochrome *c*-specific T cell proliferative line, prepared according to the method of Kimoto and Fathman (20), were cocultured at the end of a 2-wk rest period with 5×10^5 irradiated spleen cells or with varying numbers of A20.3 tumor cells for 3 d in the presence or absence of antigen. The cultures were pulsed 8 h before harvesting with 1 μCi of [^{3}H]thymidine. For simplicity, results are expressed as the cpm obtained in the presence of antigen minus the cpm obtained with media alone.

‡ Not done.

TABLE X

Three B Cell Tumors Present Antigen Poorly to Primed T Cells but Do Not Suppress the Response Elicited by A20.3 Cells

Stimulator cell	PPD-stimulated [^{3}H]thymidine uptake*		
	Stimulator cell		
	cpm		*cpm*
None	297	K46 + A20.3	124,600
A20.3	107,963	L10.A + A20.3	86,053
K46R.18	1,640	BALENLM17 + A20.3	117,242
L10.A6.2	14,106	BALENTL3 + A20.3	115,629
BALENLM17.7.2	2,969		
BALENTL3‡	126		

* 2.5×10^5 nylon-passed αIa + C-treated LN cells from B10.D2 mice primed with CFA were cocultured for 4 d with 10^4 irradiated cells from each of the tumor lines or with a mixture of 10^4 A20.3 cells plus 10^4 cells from each line in the presence of 1.25% IL-1 with or without antigen (PPD 20 μg/ml). Results are expressed as the cpm obtained in the presence of antigen minus the cpm obtained with media alone.

‡ A T cell lymphoma with the phenotype Thy-1.2$^+$ Ly-1$^-$2$^+$, TL$^+$. Two other T cell lymphomas were also tested and were also negative (not shown).

differentiation of residual Ia-positive macrophages in the T cell population because these macrophages would be F_1 in haplotype and should therefore be capable of stimulating a proliferative response to collagen as well as to GLϕ and PPD.

The second approach made use of antigen-reactive T cell lines. A BALB/c line reactive to OVA and a B10.A line reactive to pigeon cytochrome *c* were stimulated with varying numbers of A20.3 cells or control spleen cells in the presence of antigen. The data in Table IX demonstrate that as few as 10^3 A20.3 cells could effectively present OVA to the BALB/c line. However, even 10^4 A20.3 cells failed to present pigeon cytochrome *c* to the I region-disparate B10.A T cell line. Again, a good response was obtained in the absence of exogenous IL-1, although the addition of small amounts of IL-1 to this assay clearly magnified the response obtained.

K46R.18, L10A6.2, and BALENLM 17.7.2 Present Antigen Poorly but Do Not Suppress Responses Stimulated by A20.3. In Table III we showed that the M12.4 and X16C lines, like A20.3, stimulate an allogeneic MLR. These two tumors are also able to present PPD to primed T cells (Table V). Three Ia antigen-positive B cell tumors—K46R.18, L10A6.2, and BALENLM17.7.2—failed to stimulate an allogeneic MLR. The ability of these tumors to present PPD was tested in a series of experiments. The K46R.18 tumor failed to present PPD to primed T cells in four of four experiments; the BALENLM17.7.2 did produce T cell proliferation when used at the highest dose of 10^5 cells in one of three experiments, and the L10A6.2 produced modest T cell proliferation to antigen when used at the highest dose of 10^5 cells (Table X). A possible explanation for the relatively poor ability of K46R.18, L10A6.2, and BALENLM17.7.2 to present antigen was that these particular B cell tumors secreted suppressive material into the culture supernatant and thus masked or prevented a proliferative T cell response. This explanation does not appear to be correct because mixing cells from each of these three lines with A20.3 cells did not decrease the excellent proliferative response induced by the latter.

Discussion

The ability to present protein antigen or alloantigen to T cells in vitro has been shown to reside in an Ia antigen-positive, radioresistant, adherent, phagocytic, Ig^-, FcR^+ cell of the macrophage lineage (8). More recently, Steinman and his co-workers (12) have provided convincing evidence that the lymphoid dendritic cell which is present in very small numbers in mouse spleen and that differs from the macrophage in being nonphagocytic and FcR^-, is an extremely potent stimulator of alloreactive T cells, and Sunshine et al. (29) have further shown its effectiveness as an antigen-presenting cell. In addition, Chesnut and Grey (15) have reported that normal B cells can, under certain circumstances, present antigen to antigen-reactive T cells. In this paper we present data that demonstrate that several but not all of a series of Ia antigen-positive BALB/c B cell lymphomas grown in tissue culture can present alloantigens and protein antigens to alloreactive and antigen-reactive T cells. One of these tumors A20.3, was chosen for further study. Antigen presentation and allostimulation by this B cell tumor line appeared to have the same characteristics as those attributed to the conventional APC because (*a*) the tumor cells presented antigen in an MHC-restricted fashion to antigen-reactive T cells, (*b*) alloantigen presentation was blocked by the inclusion of monoclonal anti-Ia antibodies in the culture system, (*c*) tumor cells could be effectively pulsed with soluble antigen, and (*d*) on a per cell basis A20.3 appeared to be at least as powerful as conventional APC in whole spleen because 10^3 A20.3 cells were as effective in inducing proliferation as 10^5 irradiated spleen cells (Table V).

The statement that tumor cells with the characteristics of B cells can serve as APC and stimulators of alloreactive responses depends upon the demonstration that contaminating macrophages remaining in the T cell population were not the source of the antigen-presenting function. Residual Ia antigen-positive APC could also potentially explain the alloantigenic stimulation we obtained because such macrophages in the responder cell population could take up, process, and re-present shed Ia antigens from the allogeneic tumor cells. That such a phenomenon does occur for cytotoxic cells has been shown by Weinberger et al. for purified H-2 complex proteins (24).

To address this issue, three approaches were taken. First, we routinely treated the antigen-reactive and alloreactive nylon-passed T cell population with an αIa + C to deplete residual contaminating APC. The data obtained indicate that A20.3 cells induced an excellent allogeneic and antigen-specific response in such pretreated T cell populations. The effectiveness of the αIa treatment was demonstrated by the lack of an antigen-specific response from the treated T cell population in the absence of added tumor or spleen cells as APC, even in the presence of a source of exogenous IL-1. Second, we demonstrated that the inclusion of monoclonal antibodies directed against stimulator cell Ia molecules (MKD6 and 14.4.4S) could inhibit the proliferation observed; a monoclonal antibody directed against responder cell Ia molecules (10.2.16) was not inhibitory. Finally, the activation of allospecific and antigen-specific T cell lines by the A20.3 provides strong evidence that the tumor cell was itself providing the alloantigenic or protein antigen stimulus for T cell proliferation and not simply causing the differentiation or activation of residual Ia antigen-positive APC in the T cell population. Indeed, the capacity of A20.3 of $H\text{-}2^d$ haplotype to

activate primed F_1 T cells accorded with the Ir phenotype of H-2^d cells. That is, A20.3 was capable of presenting GLϕ but not collagen to F_1 T cells.

One interesting difference between the antigen-specific and allospecific responses studied in this paper was the necessity for the addition of an exogeneous source of IL-1 to obtain an allogeneic MLR. Although the addition of IL-1 clearly enhanced the proliferation generated by antigen-specific T cells, it was not necessary. A good proliferative response could be obtained in its absence. Studies by Weinberger et al. (24) suggested that a factor, presumably elaborated by the accessory cells, as well as macrophage-bound antigen, was necessary for stimulation of an alloreactive response. We have not been able to demonstrate constitutive IL-1 production by the B lymphoma cell lines, but it is possible that they do produce small amounts of IL-1 during the culture period. Alternatively, there may be another source of IL-1 in these cultures. These amounts might be sufficient to provide the second signal for antigen-reactive T cells but insufficient to trigger alloantigen-reactive T cells.

We cannot explain the relatively poor ability of three of the six BALB/c B cell tumors to either stimulate an allogeneic MLR or to present antigen. It does not appear to be related to surface Ia antigen density (K. J. Kim, unpublished observation) nor do these tumors appear to produce suppressive secretory products. The diminished capacity of these cells to present antigen even in the presence of exogeneous IL-1 also makes a simple lack of secretion of this factor unlikely as an explanation. It is possible that there is yet another factor required to trigger antigen-reactive T cells and secreted by classic APC and by three of the six B cell tumors discussed here but not secreted by the three poorly stimulatory tumors. Alternatively, a defect in taking up and presenting antigen might be responsible, although the fact that these B cells tumors are nonstimulatory in an allogeneic MLR as well as poorly stimulatory in a protein antigen-specific response makes this less likely.

A cloned and easily obtainable population of B lymphoma cells endowed with the ability to present alloantigen and protein antigen to alloreactive and antigen-reactive T cells in an MHC-restricted fashion as efficiently on a per cell basis as the conventional APC should provide a useful model system to examine the biochemical events involved in antigen uptake, processing, and I region-associated antigen presentation to long-term continuous antigen-reactive T cell lines.

Summary

Several Ia-positive BALB/c B cell tumor lines were screened for their ability to present alloantigen and protein antigens to alloreactive and antigen-reactive T cells. Of six Ia-positive tumor lines studied, three were found to be effective as antigen presenting cells (APC). Indeed, on a per cell basis, one of the stimulatory lines, A20.3, was substantially more effective than whole spleen cells. The other three lines, although Ia-positive, were nonstimulatory. A20.3 was chosen for further study. This tumor appeared to behave like the conventional APC because (*a*) the tumor cells presented alloantigen, (*b*) they presented protein antigen in an MHC-restricted fashion to both primed donor T cells and to long-term continuous T cell lines, (*c*) alloantigen presentation was blocked by the inclusion of an anti-Ia antibody in the culture system, and (*d*) A20.3 cells could be effectively pulsed with antigen, although the continuous presence of antigen in the culture system resulted in a superior response. The addition of an exogenous source of interleukin 1 proved necessary to

obtain an alloreactive but not an antigen-specific T cell response, although its inclusion did enhance the magnitude of antigen-stimulated proliferation. These tumor cells should prove useful in studying the biochemical events that occur during antigen processing and the requirements for T cell triggering by processed antigen in association with Ia molecules.

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