Brief Definitive Reports

EVIDENCE FOR A ROLE OF *Ir*-ASSOCIATED ALLOANTIGENS IN MIXED LYMPHOCYTE CULTURE STIMULATION

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(Received for publication 19 February 1974)

A variety of *Ir*-associated murine lymphoid cell surface antigens (Ia antigens), at least some of which are preferentially expressed on B cells, have recently been reported (1–3). They are determined by genetic information mapping in the *Ir* region of the mouse major histocompatibility complex (MHC). In this laboratory one such Ia antigen has been demonstrated on B cells of an $H\text{-}2^a,H\text{-}2^b$ recombinant congenic strain (B10.A(2R)) (2). No corresponding antigen has yet been demonstrated on B10.A(4R) cells. It has therefore been suggested that this Ia alloantigen of B10.A(2R) mice may be responsible for the "one-way" mixed lymphocyte culture (MLC) reaction between these two recombinant strains (2, 4). In this paper we further assess the possible role of Ia alloantigens in the MLC.

Materials and Methods

Mice.—Mice of strains B10.A/SgSn and B10.D2/nSn were obtained from Jackson Laboratories, Bar Harbor, Maine. Hybrid (B10 × B10.A)F_1 animals were produced in our own breeding colonies. B10.A(4R) and B10.A(2R) animals were obtained from breeding colonies established from stock kindly provided by Dr. J. Stimpfling, McLaughlin Institute, Great Falls, Mont.

MLC.—Mouse spleens from 10- to 20-wk old animals were teased into medium RPMI 1640 (Media Unit, NIH) supplemented with glutamine, penicillin, streptomycin, and 5% heat-inactivated fetal calf serum. MLC assays were carried out in triplicate in microtiter "V" plates in a final vol of 200 μl/well. MLC assays were performed using stimulator cells which were irradiated with 2,000 R. Control cultures consisted of 5×10^5 irradiated and 5×10^5 nonirradiated syngeneic cells. Experimental cultures consisted of 5×10^5 irradiated separated or unseparated allogeneic stimulator cells and 5×10^5 nonirradiated unseparated responder cells. For dose response curves unseparated irradiated syngeneic cells were added to irradiated allogeneic stimulator cells. The cells were incubated for 68–72 h at 37°C in an atmosphere containing 5% CO_2 and 95% air. During the last 4 h of culture, 1 μCi of [*methyl*-^{3}H]thymidine was added to each well. The cultures were then harvested and counted by the method of Rosenberg and Levy (5).

Nylon Column Fractionation.—Fractions enriched for B cells and T cells were obtained using a modification of the nylon column technique of Julius et al. (6) developed by Handwerger and Schwartz.[1] Briefly, after column incubation the T-cell-enriched subpopulation was eluted dropwise and, after extensive washing, the nylon was mechanically agitated to release the adherent B-cell-enriched subpopulation. Aliquots were assayed for cells bearing surface

[1] Handwerger, B. S., and R. H. Schwartz. 1974. Purification of murine bone marrow-derived and thymus-derived lymphocytes using nylon wool columns. Manuscript submitted for publication.

immunoglobulin using a fluorescein-conjugated rabbit antimouse Ig serum. In selected experiments, fluorescein-conjugated rabbit antimouse brain antisera was used to strain T cells.

RESULTS

In order to test the hypothesis that Ia alloantigens are responsible for MLC stimulation, we first examined the MLC reactions between B10.A(4R) and B10.A(2R) spleen cells. Our results (Table I) confirmed the unidirectional MLC reaction between these two strains which correlates with the expression of the Ia alloantigen described by Lozner et al. (2). We then proceeded to examine separate strain combinations in which we could test possible MLC stimulation due to another Ia alloantigen.

TABLE I

MLC Responses

Responder	Stimulator	Known MHC differences	No of exp.	Mean stimulation index ± SE*
B10.A	B10.D2	K, *Ir*, Ia	9	2.8 ± 0.2
(B10 × B10.A)F_1	B10.D2	K, *Ir*‡	4	1.5 ± 0.2
B10.A(4R)	B10.A(2R)	*Ir*, Ia	4	1.9 ± 0.3
B10.A(2R)	B10.A(4R)	*Ir*	4	0.9 ± 0.2

* Mean stimulation index ± SE represents the mean value ± SE obtained by comparing mean stimulation indices from replicate experiments. The stimulation index (E/C) is the ratio obtained by dividing the mean cpm of the experimental culture (E) by the mean cpm of the irradiated and nonirradiated syngeneic control culture (C).

‡ Undefined Ia differences may exist.

We chose strain combinations in which we could compare the effect on MLC stimulation of the presence or absence of the Ia alloantigen-β (1). An examination of the one-way MLC reactions of B10.A and of (B10 × B10.A)F_1 responder cells against irradiated B10.D2 stimulator cells might be expected to accomplish this comparison. While both of these responder cell populations lack the *H-2k* region specificity (*H-2.31*) present on the *H-2*^{d} stimulating cells, only the B10.A responders also lack β. In addition, the MHC of B10.A and B10.D2 strains share the same *H-2D* and Ss-Slp regions and any MLC stimulations obtained would therefore be attributable to antigens determined by genetic information in or to the left of the *Ir* region.

The mean stimulation index obtained using B10.A responder and irradiated B10.D2 stimulator cells was significantly greater ($P < 0.01$) than that obtained using (B10 × B10.A)F_1 cells and the same irradiated B10.D2 stimulator cells (Table I). The presence of the serologically detected Ia alloantigen-β thus correlates with the increased stimulation observed between B10.A responder and B10.D2 stimulator cells. It is clear, however, that even without the β-differ-

ence, (B10 × B10.A)F_1 cells are stimulated by B10.D2 stimulator cells. This may be due to Ia antigens other than β (unpublished results).

If the hypothesis that these Ia alloantigens are relevant targets in MLC stimulation is correct, then B cells should account for the majority of the stimulation of the MLC reaction in these systems. To test this hypothesis, B10.D2 and B10.A spleen cells were enriched for T or B cells by nylon column fractionation, irradiated, and used as stimulator populations for B10.A or B10.A(4R) responder cells respectively.

The B-cell-enriched fraction was much more effective as a stimulator than the T-cell-enriched cell population (Table II). Thus the majority of stimulation

TABLE II

MLC Responses Using Nylon Column Fractionated Stimulator Cells

Responder	Stimulator	Exp. no.	Control cpm + SE	Unfractionated	B-cell-enriched adherent	T-cell-enriched nonadherent	Adherent and Nonadherent
B10.A(4R)	B10.A(2R)	1	1328 ± 54	1.20*	2.60	0.90	1.50
		2	641 ± 221	2.83	5.10	1.75	3.96
		3	5357 ± 245	1.59	1.27	0.74	NT
		4	1433 ± 61	1.80	2.06	0.80	NT
Mean E/C ± SE				1.86 ± .35	2.76 ± .83	1.05 ± .24	2.73 ± 1.23
Mean % Ig +				61%	81%	8.5%	45%
B10.A	B10.D2	5	1360 ± 87	1.40	2.10	1.02	1.90
		6	2062 ± 186	1.57	2.90	1.47	2.50
		7	334 ± 10	2.59	4.80	2.00	3.10
		8	251 ± 16	3.44	6.70	1.84	5.65
Mean E/C ± SE				2.25 ± 0.48	4.13 ± 1.03	1.57 ± .22	3.29 ± 0.83
Mean % Ig +				56%	91%	10.5%	55%

* MLC responses are reported here as E/C (see legend of Table I).

in these two systems is derived from the B-cell subpopulation. It is important to note that we have used the unseparated syngeneic culture as the control in order to analyze the relative role of each subpopulation in the total unseparated MCL stimulation.

It is apparent from Table II that the B10.D2 T-cell-enriched fraction caused consistent, though low, stimulation in the B10.A responder population. To investigate the possibility that contaminant B cells were responsible for this stimulation, a dose response experiment was performed using dilutions of spleen cells as stimulators (Fig. 1). The stimulation produced by the T-cell-enriched populations can be replotted as a function of the number of known B-cell contaminants in each such stimulator population. Such points fall fairly close to the extrapolated B-cell stimulation curve in each instance, suggesting that the stimulation produced by the T-cell-enriched subpopulation may in fact have been due to B-cell contaminants.

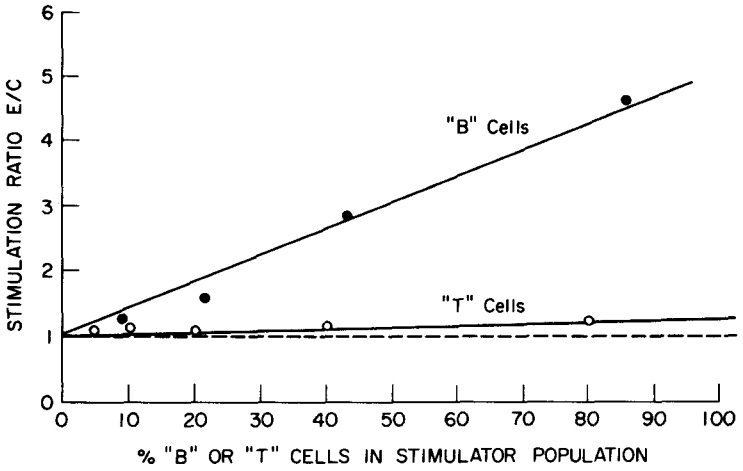

FIG. 1. Dose response curve obtained for B10.A stimulator cells using serial dilutions of either B-cell-enriched or T-cell-enriched B10.D2 stimulator populations. Stimulator populations were adjusted to 5×10^5 cells/well by the addition of irradiated syngeneic responder cells in each instance. A horizontal line indicating absence of stimulation (E/C = 1) has been added for clarity of presentation. For calculations, T cells were approximated to be 80% of the nonadherent cell population based on previous studies in which T cells in similar preparations were stained with rabbit antimouse brain sera.

DISCUSSION

Recent attempts to identify *Ir*-gene products on lymphocytes by serologic methods have led to the demonstration of several *Ir*-associated (Ia) mouse lymphocyte alloantigens (1–3). In our laboratory Ia alloantigens have so far been identified predominantly on B cells. The detection of one such alloantigen on the B cells of B10.A(2R) mice and the lack of expression of a corresponding (allelic) antigen on B10.A(4R) cells led to the hypothesis that these antigens might be responsible for the one-way MLC reaction previously observed between these two recombinant strains of congenic mice (2).

Proof of such an hypothesis would require biochemical isolation of responsible alloantigens, since genetic studies can demonstrate close association between genes determining two antigens but can never prove their identity. There are, however, certain corollaries to this hypothesis which can be tested.

The first corollary we examined is that other systems differing by serologically demonstrable Ia alloantigens should also show MLC stimulation which correlates with these antigens. The results indicate that B10.A cells respond significantly better to B10.D2 stimulator cells (β-difference) than do (B10 $\times$ B10.A)F_1 cells (no β-difference). This increased stimulation suggests that β, like the Ia alloantigen of B10.A(2R), may be a target of the MLC reaction.

The second corollary tested is that if Ia alloantigens are responsible for MLC

stimulation, B cells should account for the majority of MLC stimulation in unseparated allogenic cultures. The results are consistent with this prediction.

The stimulation of B10.A responder cells by the T-cell-enriched subpopulation of B10.D2 stimulator cells might be explained by several alternative possibilities. T-cell-enriched subpopulations may recognize antigenic determinants on responder cells and by several possible mechanisms cause thymidine incorporation in the responder population (7). It is also possible that a small subpopulation of B10.D2 T cells bear Ia antigens which stimulate the MLC response. A third possible explanation is that either or both subpopulations are modified in some manner by the separation procedure and/or irradiation and that this affects their ability to stimulate an MLC response. The most trivial explanation is that contaminant B cells in the T-cell-enriched subpopulation are responsible for the MLC stimulation. Dose-response studies in the B10.A-B10.D2 system support this possibility.

These studies have been carried out entirely between congenic-resistant mouse strains differing only at the *H-2* complex and with demonstrable Ia B-cell alloantigens. These results, therefore, do not preclude the possibility that other alloantigens (*H-2* linked or non-*H-2* linked) with different tissue distributions may also be effective targets of MLC stimulation. Furthermore, we do not wish to imply that allogeneic T cells per se are incapable of stimulating an MLC response. In fact, preliminary experiments in our laboratory indicate that certain allogeneic T-cell populations can cause MLC stimulation relative to syngeneic T-cell controls. However, in these systems, using the unseparated syngeneic control, B cells appear to provide the major source of stimulation.

Ongoing serologic studies in our lab and elsewhere indicate that there are many more Ia alloantigens than were originally suspected. It is possible that each Ia antigen may contribute to MLC stimulation. If this proves to be so, then the necessity of an "LD" difference between strains for stimulation in the MLC previously reported by Bach et al. (4) may be explained by the attendant *Ir*-associated serologically demonstrable Ia alloantigens.

The demonstration that these Ia antigens may be important stimulators of the MLC reaction does not, of course, indicate their natural biologic significance. A possible mechanism by which B-cell surface antigens could play a role in a T-cell-dependent immune response might be for those antigens to be involved in the T-cell-B-cell interaction requisite to that response. This possibility is supported by recent work of Katz et al. (8). It seems possible that the Ia alloantigens may be the receptors which permit physiologic T-cell-B-cell cooperation. One might then postulate that T cells ordinarily interact with B cells via an Ia receptor on the cell surface. In an allogeneic system (i.e., Ia difference) this interaction could lead to a proliferative response (MLC) whereas in a syngeneic system (i.e., Ia identical) it might permit physiologic functional interaction and cooperation.

The authors wish to thank Dr. William Terry and Dr. William Paul for helpful discussions, Dr. Rose Lieberman who kindly provided B10.A(4R) mice, Drs. Ronald Schwartz and Michael Karpf for obtaining some of our MLC data, Mr. James Cone and Mr. John Williams for excellent technical assistance, and Mr. Walter Lyles and Mr. Francis Jones for animal care.

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